UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
CENTRO DE BIOTECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

Epigenética em tumores pediátricos do sistema nervoso: efeito do ácido valpróico em células tronco de meduloblastoma

Natália Hogetop Freire

Porto Alegre, novembro de 2022

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Lista de abreviaturas e siglas

BMI1: *BMI1 proto-oncogene, polycomb ring finger*

CD133: *Prominin 1*

CD90: *Thy-1 cell surface antigen*

CSC: *Cancer stem cells*

CTH: Células tronco hematopoiéticas

CTNNB1*: Catenin Beta 1*

CTT: Células tronco tumorais

DIPG: Glioma pontino intrínseco difuso

DNA: Ácido desoxirribonucleico

ENO2: Enolase 2

GABA: Ácido gaba-aminobutírico

GCN5: *Lysine acetyltransferase 2A*

GLI: *GLI family zinc finger*

GNAT: *Glycine-N-acyltransferase like 1*

H3K27: Histona 3 lisina 27

H3K4: Histona 3 lisina 4

H3K9: Histona 3 lisina 9

HAT: Histona acetiltransferase

HDAC: Histona deacetilase

HDACi: Inibidor de histona deacetilases

HES1: *Hes family BHLH transcription factor 1*

HSP90: *Heat shock protein 90 alpha family class A member 1*

LMA: Leucemia mielóide aguda

MB: Meduloblastoma

MYC: *MYC proto-oncogene, BHLH transcription factor*

NaB: Butirato de sódio

NAD: Nicotinamida adenina dinucleotídeo

NANOG: Nanog homeobox

NB: Neuroblastoma

NMDA: N-metil D-aspartato

NMYC: *MYCN proto-oncogene, BHLH transcription factor*

NOD/SCID: *Nonobese diabetic/severe combined immunodeficiency*

NOTCH1: *Notch receptor 1*

OCT4: *POU class 5 homeobox 1*

p21: Inibidor de quinase dependente de ciclina 1

p27: Inibidor de quinase dependente de ciclina 1B

p300/CBP: *E1A binding protein P300*

p53: Proteína de tumor p53

pGM: Gliomas pediátricos

pRB: Proteína retinoblastoma

RBFOX3: *RNA binding fox-1 homolog 3*

RNA: Ácido ribonucleico

SHH: *Sonic hedgehog*

SIRT: Sirtuinas

SMO: *Smoothened, frizzled class receptor*

SNC: Sistema nervoso central

SOX2: *SRY-Box transcription factor 2*

SUFU: *SUFU negative regulator of hedgehog signaling*

TSA: Tricostatina A

TUBB3*: Tubulin Beta 3 class III*

VPA: Ácido valpróico

WNT: Wingless

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RESUMO

Meduloblastoma, neuroblastoma e glioma pediátricos representam aproximadamente 30% dos casos de câncer pediátricos. Estes tumores pediátricos do sistema nervoso são considerados a primeira causa de morte relacionada à doença na população pediátrica. Uma das principais características em comum entre os tumores pediátricos do sistema nervoso é a presença de uma subpopulação de células altamente tumorigênica que apresenta características similares às células tronco, conhecida como células tronco tumorais (CTT). Esta população de células contribui para iniciação, progressão tumoral e resistência ao tratamento. Modificações em histonas, metilação do DNA, remodeladores da cromatina e microRNAs são capazes de regular o estabelecimento e a manutenção da população tronco tumoral. Moduladores epigenéticos, em ênfase aqueles capazes de alterar o perfil de acetilação de histonas, como Ácido Valpróico (VPA), são considerados uma potencial estratégia contra as células tronco tumorais. Neste trabalho, foi encontrado que o VPA reduz a viabilidade de CTT e tem efeito em vias de sinalização relacionadas ao processo de diferenciação neuronal e genes de *stemness.* Nossos resultados sugerem um relação entre a capacidade de moduladores epigenéticos de reduzirem a tumorigenicidade e alterar o estado da cromatina permitindo a regulação de genes importantes para manutenção das CTT, se tornando terapias em potencial para o tratamento de tumores pediátricos do sistema nervoso como meduloblastoma, neuroblastoma e glioma pediátrico.

Palavras-chave: Meduloblastoma, Neuroblastoma, Glioma pediátrico, células tronco tumorais, epigenética.

ABSTRACT

Medulloblastoma (MB), neuroblastoma (NB), and pediatric glioma (pGM) account for almost 30% of all cases of pediatric cancers and are the leading cause of death in this population. A common feature between these pediatric nervous system tumors is the presence of a highly tumorigenic subpopulation of cells, which presents stem cell-like features, known as cancer stem cells (CSC). This subpopulation of cells plays a role in initiation, progression, and resistance to treatment of pediatric nervous system tumors. Histone modification, DNA methylation, chromatin remodeling, and microRNA regulation are major drivers for the establishment and maintenance of CSC, and histone deacetylase (HDAC) inhibitors are considered a therapeutic strategy to target this cell population. Valproic acid (VPA) is an epigenetic modulator capable of inhibiting HDAC. Here we found that VPA decreases MB cell and CSC viability through modulation of neuronal differentiation and stemness pathways. Our results indicate that epigenetics can reduce tumorigenicity by altering the chromatin state and therefore are potential therapies to pediatric nervous system tumors.

Key words: Medulloblastoma, Neuroblastoma, pediatric Glioma, Cancer stem cells, epigenetic modulators.

INTRODUÇÃO

Tumores pediátricos do sistema nervoso

Tumores de sistema nervoso são considerados a primeira causa de morte relacionada a câncer em crianças e adolescentes. Os tumores de sistema nervoso mais frequentes são Meduloblastoma (MB), Neuroblastoma (NB) e Gliomas pediátricos (pGM), representando aproximadamente 30% dos casos de câncer pediátrico (SIEGEL *et al*., 2022). NB é um tumor derivado da crista neural que atinge o sistema nervoso periférico (JOHNSEN *et al*., 2019). Gliomas pediátricos são um grupo de tumores de sistema nervoso central composto por gliomas de baixo grau, gliomas de alto grau e glioma pontino intrínseco difuso (DIPG). Este grupo heterogêneo de tumores afeta múltiplas regiões do cérebro (FUNAKOSHI *et al*., 2021). Meduloblastoma é o tumor que ocorre no cerebelo e é o tumor maligno do sistema nervoso central mais frequente em crianças e adolescentes (NORTHCOTT *et al*., 2019).

Meduloblastoma

Meduloblastoma (MB) é o tumor maligno de sistema nervoso central mais frequente em pacientes pediátricos, apresentando taxa de sobrevida global de aproximadamente 75% (ORR, 2020). Segundo estimativas, 70% dos casos de MB ocorrem em crianças com idade inferior a 10 anos, sendo os picos de incidência entre 1 a 4 anos e 5 a 9 anos de idade (OSTROM *et al*., 2019). MB é considerado um tumor embrionário que acomete o cerebelo e evidências apontam que a origem celular desse tumor inclui precursores de neurônios cerebelares, células tronco neurais e progenitores neurais (GIBSON *et al*., 2010) (JURASCHKA & TAYLOR, 2019).

Estudos genômicos, epigenômicos e transcriptômicos possibilitaram o estabelecimento de quatro subgrupos moleculares de MB: *Wingless* (WNT), *Sonic Hedgehog* (SHH), Grupo 3 e Grupo 4. Os subgrupos apresentam diferentes perfis transcricionais, alterações genéticas e prognóstico clínico (Figura 1) (TAYLOR *et al*., 2012).

Tumores do subgrupo WNT correspondem a cerca de 10% dos diagnósticos de MB, apresentam o melhor prognóstico entre os subgrupos, com rara presença de metástase e taxas de sobrevivência em 5 anos excedendo 95% (FATTET *et al*., 2009) (TAYLOR *et al*., 2012).

Alterações associadas à via de sinalização *Wingless* proporcionaram o nome para o subgrupo, sendo a principal delas, presente em 85-90% dos casos, a mutação no exon 3 do gene *CTNNB1* (gene que codifica a proteína beta-catenina). Esta mutação permite a estabilização de beta-catenina resultando na expressão constitutiva da via de sinalização WNT e propiciando o crescimento tumoral (THOMPSON *et al*., 2006) (NORTHCOTT *et al*., 2017).

O subgrupo SHH representa 30% dos casos de MB e apresenta um prognóstico intermediário que está associado ao status de TP53. Pacientes com SHH-TP53-WT (*Wild type*) possuem uma taxa de sobrevivência de aproximadamente 80%, enquanto aqueles com SHH-TP53-MUT (mutada) apresentam taxas menores que 50% (RAMASWAMY *et al*., 2016). SHH foi denominado desta forma devido às mutações frequentemente serem encontradas em genes participantes da via de sinalização *Sonic Hedgehog*, como por exemplo: PTCH1, SUFU, SMO, GLI1 e GLI2 (TAYLOR *et al*., 2012). Além disso, tumores SHH possuem heterogeneidade quanto a biologia e prognóstico clínico, e estudos do perfil de metilação do DNA e expressão gênica identificaram 4 subtipos de meduloblastoma SHH: SHH α, SHH β, SHH γ, e SHH δ. Os subtipos de SHH retêm valor prognóstico porque apresentam diferentes taxas metastáticas, alterações genéticas e idade no diagnóstico (CAVALLI *et al*., 2017).

Tumores do grupo 3 correspondem a 25% dos diagnósticos de MB e apresentam o pior prognóstico entre os subgrupos, uma vez que 40-45% dos pacientes apresentam metástases no momento do diagnóstico e as chances de sobrevivência após 5 anos encontram-se abaixo de 60% (TAYLOR *et al*., 2012). Mutações somáticas são incomuns neste subgrupo, de forma que uma das características marcantes do grupo 3 são amplificações do gene MYC, que é considerado um fator de risco que contribui para progressão dessa neoplasia (NORTHCOTT *et al*., 2012).

O grupo 4 representa cerca de 35-40% dos casos de MB e, apesar de ser uma porcentagem significativa dos diagnósticos, as características biológicas desse subgrupo ainda são pouco entendidas (TAYLOR *et al*., 2012). Assim como ocorre no grupo 3, tumores do grupo 4 raramente apresentam mutações somáticas, e uma das alterações mais frequentes é a instabilidade cromossômica que ocorre no isocromossomo 17q (KOOL et al., 2012). Esse subgrupo possui um prognóstico intermediário, com taxas de metástases em

aproximadamente 35% dos casos e tumores que não apresentam altos níveis de expressão de fatores de risco MYC e MYCN (NORTHCOTT *et al*., 2012) (RAMASWAMY *et al*., 2013).

	Subgroup	WNT	SHH	Group 3	Group 4
Clinical Characteristics	% of Cases	10	30	25	35
	Age at Diagnosis	Child, Adult	Infant, Child, Adult	Infant, Child	Child
	Gender Ratio (M:F)	1:1	1:1	2:1	3:1
	Anatomic Location				
	Histology	Classic, Rarely LCA	Desmoplastic, Classic, LCA	Classic, LCA	Classic, LCA
	Metastasis at Diagnosis (%)	5-10	15-20	40-45	35-40
	Recurrence Pattern	Rare; Local or metastatic	Local	Metastatic	Metastatic
	Prognosis	Very good	Infants good, others intermediate	Poor	Intermediate
Molecular Characteristics	Proposed Cell of Origin	Progenitor cells in the lower rhombic lip	Granule precursors of the external granule layer	Neural stem cells	Unipolar brush cells
	Recurrent Gene Amplifications	-	*MYCN* *GLI1* or *GLI2*	*MYC* *MYCN* *OTX2*	*SNCAIP* *MYCN* *OTX2* *CDK6*
	Recurrent SNVs	*CTNNB1* *DDX3X* *SMARCA4* *TP53*	*PTCH1* *TERT* *SUFU* *SMO* *TP53*	*SMARCA4* *KBTBD4* *CTDNEP1* *KMT2D*	*KDM6A* *ZMYM3* *KTM2C* *KBTBD4*
	Cytogenetic Events (Gain / Loss)	Loss: 6	Gain: 3q, 9p; Loss: 9q, 10q, 17p	Gain: 1q, 7, 18; Loss: 8, 10q, 11, 16q; i17q	Gain: 7, 18q; Loss: 8, 11p, X; i17q
	Other Recurrent Genetic Events	-	-	*GFI1* and *GFI1B* enhancer hijacking	*PRDM6*, *GFI1*, and *GFI1B* enhancer hijacking

Age: Infant Child Adult

Figura 1: Caracterização clínica e molecular dos subgrupos de meduloblastoma (JURASCHKA & TAYLOR, 2019).

Em geral o prognóstico de MB pode ser associado a fatores de risco como idade, tamanho do tumor e presença de metástase, assim como o subgrupo molecular ou histológico, que pode ser clássico, desmoplásico ou anaplásico (JURASCHKA & TAYLOR, 2019). Os tratamentos empregados para este tumor são: ressecção cirúrgica, radioterapia e quimioterapia (THOMPSON *et al*., 2016). Apesar dos tratamentos auxiliarem nas taxas de sobrevida, pacientes pediátricos de MB apresentam uma baixa qualidade de vida devido ao efeito dos tratamentos no desenvolvimento do cérebro. Aproximadamente 25% dos

pacientes que realizaram tratamento para MB apresentam dificuldades na fala e audição, e perda de capacidade neuro cognitiva (CHEVIGNARD *et al*., 2017). Além disso, a ocorrência de metástases e casos de recorrência tumoral, são considerados um dos principais desafios no tratamento do meduloblastoma (KUMAR *et al*., 2017).

Células tronco tumorais

Tumores são formados por uma população heterogênea de células que apresentam diversidade fenotípica que incluem diferentes taxas de crescimento, expressão de marcadores, alterações genéticas e epigenéticas, e sensibilidade ao tratamento (PRASETYANTI & MADEMA, 2017). Existem dois modelos para compreender a heterogeneidade intratumoral. O modelo de evolução clonal propõe que células geneticamente instáveis acumulam alterações genômicas e genéticas possibilitando a formação e manutenção de células mais resistentes e agressivas, promovendo assim a formação de um tumor heterogêneo (GERDES *et al*., 2014). O modelo de células tronco tumorais propõe a existência de uma subpopulação de células que apresentam características similares às células troncos normais (*stemness*), como auto renovação e potencial de diferenciação em células com fenótipos distintos, proporcionando a formação de um tumor heterogêneo (BECK & BLANPAIN, 2013). Além disso, células tumorais apresentam plasticidade celular, sendo capazes de alterar seu fenótipo de acordo com estímulos do microambiente, possibilitando que uma célula transite de um estado tronco para um estado diferenciado (CABRERA et al., 2015) (RICH, 2016) (Figura 2). Dessa forma, o modelo de evolução clonal e de células tronco tumorais não são mutuamente exclusivos.

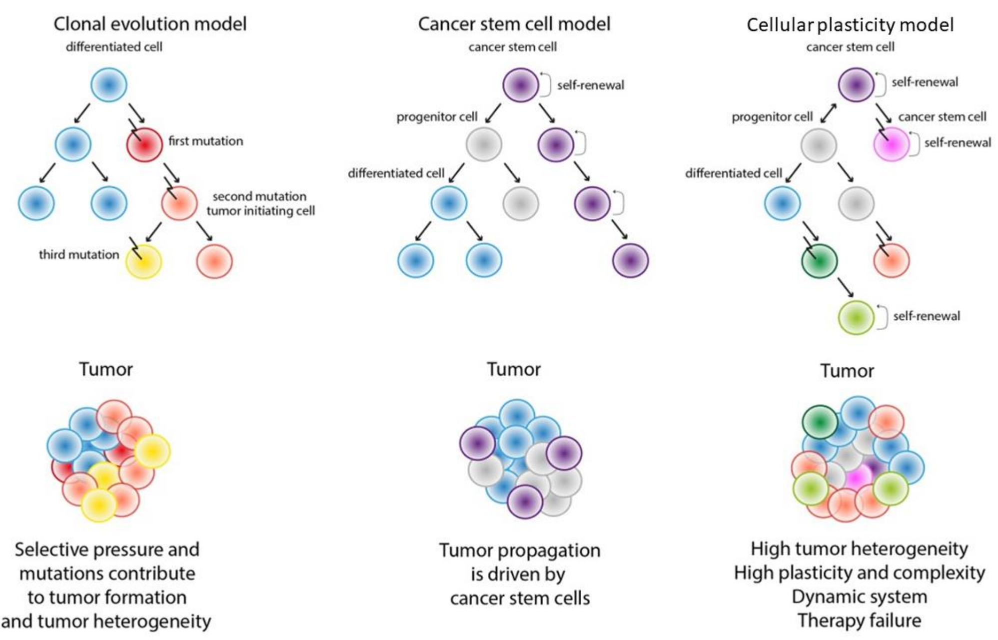

Figura 2: Teorias de heterogeneidade tumoral. Adaptado de (CORRÒ & MOCH, 2018).

Apesar de não termos uma definição da origem da heterogeneidade tumoral, evidências sugerem que células com característica tronco podem ser encontradas em todos os estágios da progressão tumoral, e que são estas células que apresentam resistência aos tratamentos antineoplásicos, possibilitando a recidiva tumoral (AYOB & RAMASAMY, 2018). Células tronco e células tronco tumorais (CTT) compartilham similaridades, dessa forma, é possível caracterizar e isolar CTT com marcadores de superfície de células tronco, como por exemplo CD133, CD44, CD90, além de ser possível isolar uma subpopulação de células que podem ser enriquecidas in vitro e in vivo (BECK & BLANPAIN, 2013).

O estudo pioneiro sobre células tronco tumorais utilizou conhecimento de células tronco hematopoiéticas (CTH) para isolar e identificar células tronco tumorais em leucemia mielóide aguda humana (LMA). Este estudo demonstrou que apenas uma subpopulação de células, que apresentava a expressão de marcadores de CTH (CD34+CD38−), foi capaz de originar e propagar leucemia em camundongos NOD/SCID. Essa população de células foi nomeada células iniciadoras de leucemia ou célula tronco de leucemia (LAPIDOT et al., 1994) (BONNET & DICK, 1997). O primeiro estudo que foi capaz de caracterizar CTT em tumores sólidos também utilizou esta estratégia, demonstrando que células de câncer de mama que expressam CD44+CD24− possuem capacidade de propagar o tumor aos serem transplantadas em camundongos imunodeficientes (AL-HAJJ et al., 2003).

Em tumores do sistema nervoso central (SNC), como glioblastoma e meduloblastoma, estudos demonstraram a presença de células com capacidade de diferenciação em células neurais distintas. Além disso, foi encontrado que tumores do SNC e neuro esferas derivadas destes tumores expressam proteínas associadas a células tronco neurais como por exemplo: CD133, SOX2 e BMI1 (HEMMATI *et al*., 2013) Dessa forma, é sugerida uma participação significativa de células tronco tumorais no desenvolvimento de tumores do SNC. Em MB, foi identificado que apenas uma porção de células do tumor apresenta expressão do marcador de células tronco CD133, reforçando a ideia que existe uma população de células tronco tumorais nessa neoplasia (SINGH *et al*., 2003). Além disso, estudos correlacionaram que vias de sinalização relevantes para manutenção de CTT também tem um papel crucial em MB, indicando que a heterogeneidade do MB pode ser decorrente de uma subpopulação de células que apresentam fenótipo similar a células tronco (FAN & EBERHART, 2008).

Durante a iniciação e progressão tumoral, células tumorais mantêm altas taxas de crescimento, ativam vias que permitem o aumento de vascularização do tumor, e aumentam taxas de migração (HANAHAN & WEINBERG, 2011). Estes processos celulares podem ser associados a presença de células tronco tumorais, visto que elas são capazes de modular o microambiente ativando a angiogênese e regulando a transição epitélio-mesênquima, propiciando um aumento nas taxas de migração celular e de distribuição de nutrientes e oxigênio para o tumor (BAO *et al*., 2006) (NGUYEN *et al*., 2012). Assim, a presença de células com características tronco aumenta as chances de formação de metástases, uma vez que CTTs têm maiores taxas de migração e de invasão e são capazes de ativar vias de angiogênese.

CTT também podem ser relacionadas a mecanismos de resistência e recorrência tumoral, visto que quimioterapias convencionais e radioterapias tem taxas de eficiência reduzidas nesta população de células. Estudos apontam que transportadores de drogas encontram-se superexpressos em CTT, diminuindo os níveis intracelulares dos agentes terapêuticos e, por consequência, reduzindo a eficiência do tratamento (BLEAU *et al*., 2009) (RIZZO *et al*., 2011). Em relação a radioterapias, sabe-se que a eficácia de terapias radio-ionizantes é influenciada pelo estágio do ciclo celular (PAWLIK & KEYOMARSI, 2004) e que CTT têm baixas taxas de progressão do ciclo celular e são, em sua maioria, quiescentes, dessa forma, radioterapias possuem baixo efeito nesta população de células (MOORE &

LYLE, 2011). Assim, pode-se perceber que os mecanismos associados à CTT influenciam a progressão, resistência e recorrência tumoral.

Modificações epigenéticas

Modificações epigenéticas é o termo utilizado para se referir a mecanismos que alteram a expressão gênica sem que haja alterações no DNA. Alterações epigenéticas são hereditárias e reversíveis e, em geral, são capazes de alterar o perfil transcricional ao modificar o estado da cromatina (DAWSON & KOUZARIDES, 2012). A cromatina é formada por repetições de octâmeros de histonas (H2A, H2B, H3 e H4) envolvidos por DNA, denominados nucleossomos. As modificações em histonas, metilação do DNA e remodeladores da cromatina são os principais mecanismos responsáveis pela arquitetura da cromatina, visto que são capazes de alterar a cromatina de um estado mais condensado e com atividade transcricional limitada (heterocromatina) para um estado menos condensado com maior atividade transcricional (eucromatina) (ZHAO *et al.*, 2021).

As histonas são proteínas que possuem uma estrutura globular e uma região N-terminal capaz de sofrer modificações covalentes. Modificações em histonas incluem acetilação, metilação, fosforilação, ubiquitinação e ADP-ribosilação (ZHAO & SHILATIFARD, 2019). Dentre estas modificações, a acetilação é uma das modificações mais estudadas no câncer. A acetilação dos resíduos de lisina na região N-terminal das histonas reduz a afinidade entre as histonas e DNA, permitindo a abertura da cromatina e auxiliando na funcionalidade de proteínas envolvidas na transcrição gênica (NIGHTINGALE *et al.*, 2006). A acetilação de histonas é resultado de um processo dinâmico regulado por histonas acetiltransferases (HAT) e histonas desacetilases (HDAC). A atividade de HAT é responsável pela abertura da cromatina e as HDACs são capazes de reduzir os níveis de acetilação, condensando a cromatina e reduzindo a transcrição (YANG & SETO, 2007). Existem três principais famílias de HATs: GNAT, GCN5 e p300/CBP, e estas agem preferencialmente em histonas, mas também podem catalisar a acetilação direta de proteínas supressoras tumorais ou oncogenes como por exemplo p53, RB, and MYC (SINGH *et al.*, 2010) (MARMORSTEIN & ZHOU, 2014). Em relação às HDACs, existem quatro principais famílias: Classe I (HDACs 1, 2, 3, and 8), que são expressas no núcleo; Classe II (HDACs 4, 5, 6, 7, 9 and 10), que possuem expressão tecido específica e podem ser translocadas entre o núcleo e o citoplasma; Classe III, também conhecidas como sirtuinas

(SIRT1-7), que apresentam um mecanismo dependente de NAD+; e Classe IV (HDAC 11) que foi descoberta recentemente e possui apenas um membro em sua classificação (ZHAO & SHILATIFARD, 2019). Assim como HATs, HDACs também são capazes de atuar em proteínas como p53, HSP90, β-catenina (SINGH *et al.*, 2010).

Tumores pediátricos, comparados a tumores adultos, apresentam baixa taxa de mutações genéticas. Dentre as mutações encontradas em tumores pediátricos, alterações em genes envolvidos na maquinaria epigenética são as mais frequentes (HUETHER *et al.*, 2014) (GRÖBNER *et al.*, 2018). Em MB, o padrão de alterações em histonas demetilases, acetil transferases e modificadores da cromatina auxiliam na estratificação dos subgrupos moleculares. No subgrupo WNT, foi encontrado mutações em remodeladores da cromatina responsáveis pela ativação de genes da via WNT (ROBINSON *et al.*, 2012). Em SHH, foi possível associar alterações somáticas e a superexpressão de HATs (NORTHCOTT *et al.*, 2017). As mutações em membros da família histonas lisinas demetilases são exclusivas dos subgrupos 3 e 4, indicando um perfil de metilação alterado nestes subgrupos de MB (NORTHCOTT *et al.*, 2009). Além disso, o padrão de acetilação da lisina 27 da histona 3 (H3K27) e mono metilação da lisina 4 da histona 3 (H3K4) auxilia na distinção do subgrupo 3 de MB (ROBINSON *et al.*, 2012). No contexto de células tronco tumorais, estudos indicam que a regulação de HAT e HDAC e, por consequência, o perfil de acetilação de histonas é capaz de modular vias associadas a *stemness* (LIU *et al.*, 2017), assim, inibidores capazes de alterar mecanismos epigenéticos chaves para CTT de MB se tornam alvos terapêuticos relevantes.

Ácido Valpróico

Ácido Valpróico (VPA, ácido 2-propilpentóico) é um ácido graxo utilizado como anticonvulsivante de amplo espectro, podendo ser indicado para tratamento de epilepsia, enxaqueca, dor neuropática e estabilizador de humor em casos psiquiátricos (CHATEAUVIEUX *et al.*, 2010). O VPA é capaz de atravessar a barreira hematoencefálica e é considerado seguro para pacientes pediátricos, inclusive aqueles que apresentam sintomas devido a presença de tumores de sistema nervoso (VAN BREEMEN *et al.*, 2007) (WOLFF et al., 2008). Os primeiros mecanismos de ação atribuídos ao VPA incluem:

potencializar a atividade do Ácido gaba-aminobutírico (GABA) (MESDJIAN *et al*., 1982), atenuar a ativação de receptores de NMDA (GEAN *et al*., 1994) e de canais dependentes de voltagem (VANDONGEN *et al*., 1986). Em 2001, foi descoberto que VPA tem efeito como inibidor de histona deacetilase (HDACi) (GÖTTICHER *et al*., 2001) (PHIEL *et al*., 2001). VPA inibe as HDACs de classe I (HDAC1, 2 e 3) e IIa (HDAC4, 5 e 7). VPA não é capaz de modular HDAC6, 8 e 10 e já foi visto que HDAC9 e 11 são ativadas por VPA (BRADBURY et al., 2005) (CHATEAUVIEUX *et al*., 2010).

Em MB, VPA possui efeito antiproliferativo dose dependente e é capaz de alterar mecanismos associados a progressão do ciclo celular, apoptose e senescência (LI *et al*., 2005). Modelos *in vivo* utilizando VPA também demonstram resultados promissores em MB. Camundongos com xenoenxerto ortotópico intracerebelar tratados com VPA tiveram uma maior sobrevida, acompanhada de uma redução do tamanho tumoral, menores taxas de angiogênese e um perfil de diferenciação aumentado (SHU *et al*., 2006). Existe uma quantidade limitada de estudos que abordem o papel de VPA em CTT, entretanto, foi encontrado que em CTT de glioblastoma, VPA é capaz de alterar a taxa de proliferação ao modular a expressão CD133, Nanog e OCT4. A redução de genes de *stemness* é acompanhada pelo aumento da expressão de marcadores de diferenciação neural, indicando que VPA é capaz de alterar a manutenção de CTT e induzir um perfil menos indiferenciado nestas células (ALVAREZ *et al*., 2015). Até o momento, não existem estudos demonstrando o efeito de VPA em CTT de MB, desta forma o presente trabalho busca elucidar o papel de VPA em vias de s*temness* e de diferenciação neural e sua contribuição para manutenção do estado tronco em MB.

HIPÓTESE GERAL

Visto que:

- Células tronco tumorais contribuem para resistência e recidiva tumoral (PRASETYANTI & MEDEMA, 2017);

- O desenvolvimento e progressão de MB está associado à presença de CTT (HEMMATI *et al*., 2003);

- Mecanismos epigenéticos encontram-se desregulados em MB e CTT (TOH *et al*., 2017) (ROUSSEL & STRIPAY, 2018);

- Moduladores epigenéticos são propostos como alvos terapêuticos para CTT ao serem capazes de induzir diferenciação (ABBALLE & MIELE, 2021);

A hipótese desse trabalho é que o modulador epigenético ácido Valpróico será capaz de alterar a manutenção do estado tronco ao regular a inibição de vias de *stemness* e a ativação de diferenciação neural em MB.

OBJETIVOS

Avaliar o efeito da modulação epigenética de VPA em vias de *stemness* e diferenciação neural de meduloblastoma humano.

Objetivos específicos:

CAPÍTULO I

i. Revisar os mecanismos epigenéticos envolvidos na regulação de células tronco tumorais de tumores pediátricos do sistema nervoso;

CAPÍTULO II

i. Avaliar curvas dose resposta de VPA nas células de MB D283 e Daoy;
ii. Caracterizar a expressão de marcadores de *stemness* Nestina, SOX2 e NOTCH1 em CTT de MB;
iii. Avaliar o efeito de antiproliferativo de VPA em CTT e sua capacidade de impedir a à indução de CTT;
iv. Determinar se a modulação de VPA reduz a atividade de HDAC e induz um aumento no perfil de acetilação de histona 3 lisina 9 (H3K9) em células e CTT de MB;
v. Analisar a modulação de VPA na progressão do ciclo celular em células e CTT de MB;
vi. Avaliar a expressão de genes de *stemness* SOX2, Nestina e NOTCH1 e marcadores de diferenciação neural TUBB3, RBFOX3 e ENO2 após a modulação de VPA em células e CTT de MB;

CAPÍTULO I

Targeting the Epigenome of Cancer Stem Cells in Pediatric Nervous System Tumors

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Targeting the epigenome of cancer stem cells in pediatric nervous system tumors

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Abstract

Medulloblastoma, neuroblastoma, and pediatric glioma account for almost 30% of all cases of pediatric cancers. Recent evidence indicates that pediatric nervous system tumors originate from stem or progenitor cells and present a subpopulation of cells with highly tumorigenic and stem cell-like features. These cancer stem cells play a role in initiation, progression, and resistance to treatment of pediatric nervous system tumors. Histone modification, DNA methylation, chromatin remodeling, and microRNA regulation display a range of regulatory activities involved in cancer origin and progression, and cellular identity, especially those associated with stem cell features, such as self-renewal and pluripotent differentiation potential. Here, we review the contribution of different epigenetic mechanisms in pediatric nervous system tumor cancer stem cells. The choice between a differentiated and undifferentiated state can be modulated by alterations in the epigenome through the regulation of stemness genes such as CD133, SOX2, and BMI1 and the activation neuronal of differentiation markers, RBFOX3, GFAP, and S100B. Additionally, we highlighted the stage of development of epigenetic drugs and the clinical benefits and efficacy of epigenetic modulators in pediatric nervous system tumors.

Keywords Epigenetic · Medulloblastoma · Neuroblastoma · Pediatric glioma · Cancer stem cells

Introduction

Cancer is the leading cause of disease-related death worldwide in children and adolescents [1]. Identification of genomic alterations and the underlying mechanisms of cancer progression in pediatric malignancies has revealed new opportunities for developing treatment strategies specific for childhood cancers. While adult tumors seem to be driven mostly by accumulating genetic alterations, recent sequencing studies have demonstrated that pediatric tumors have a significantly lower mutational rate [2, 3]. In addition, mutations found in pediatric cancers are notably different from those in adult tumors [4]. In many pediatric cancers, the most frequent mutations are associated with genes that encode proteins involved in epigenetic regulation [5].

Alterations in epigenetic mechanisms seem to contribute to the origin, cellular phenotype, and progression of nervous system tumors, a group of diseases that account for almost 30% of all cases of pediatric cancers [6, 7]. Pediatric nervous system cancer appears to originate from stem or progenitor cells [8, 9], and some characteristics of stem cells, such as self-renewal and pluripotent

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differentiation potential, are crucially regulated by epigenetic mechanisms [10].

Remarkably, several studies have shown that a highly tumorigenic subpopulation of cells, which presents stem cell-like features, is a key component of nervous system tumors, including medulloblastoma (MB), neuroblastoma (NB), and pediatric glioma [11–13]. MB is the most common malignant type of brain cancer in pediatric patients. It arises in the cerebellum and is currently classified into four distinct molecular subgroups with twelve molecular subtypes [14]. NB is a peripheral nervous system tumor and the most common type of extracranial solid cancer occurring in children [15]. Pediatric gliomas are a group of central nervous system tumors that can be divided in low-grade gliomas (LGG) or glioneuronal tumors classified by the World Health Organization (WHO) as grade I or II, high-grade glioma (HGG) (WHO grade III or IV), and diffuse intrinsic pontine glioma (DIPG). This heterogeneous group of tumors can affect multiple brain regions [16]. Although there are clear pathological differences among these cancer types, modulation of the epigenetic machinery is a potential common therapeutic target [17].

The presence of cells that express stemness genes and feature stem cell properties are now recognized as major contributors for the initiation, progression, and resistance to treatment of pediatric nervous system tumors. Studies in MB and glioblastoma (GBM) models, including tumor-derived spheres, show that the expression of genes such as Prominin 1 (PROM1) (also known as CD133*),* SRY-Box Transcription Factor 2 (SOX2), and Proto-Oncogene, Polycomb Ring Finger *(BMI1)* contributes to the initiation and maintenance of an undifferentiated state [11]. Sphere forming assays have been used to study cancer stem-like cells, and the culture conditions permit the enrichment of cells with stem cell features such as self-renewal and differentiation capability [18]. Supporting this view, Singh and colleagues [19] showed that a relatively small number of CD133 + cells, but not a population of CD133- cells, is sufficient for the formation of human MB or GBM in NOD-SCID mice, in comparison with CD133- population, indicating that CD133 expression is a marker for brain tumor stem cells capable of cancer initiation. Aggressive NB tumors also show stem cell features, such as expression of stem cell markers CD133 and Notch Receptor 1 (NOTCH1) [20]. In addition, Pandian et al. [21] demonstrated, in a metastatic NB model, that the expression of stemness genes, such as SOX2 and Nanog Homeobox (NANOG), contributes to NB plasticity and aggressiveness. In this review, we highlight the role of different epigenetic regulatory mechanisms and some of the main effects of epigenetic-modulating agents in pediatric nervous system tumor stem cells (Fig. 1).

Histone acetylation

Acetylation of lysine residues at histone tails can remove positive charges, thus reducing the affinity between histones and deoxyribonucleic acid (DNA). Therefore, histone acetylation facilitates chromatin accessibility, serving as a key helper for the recruitment of proteins involved in gene transcription [22]. Histone acetylation is the result of a dynamic process regulated by histone acetyltransferases (HATs) and deacetylases (HDACs). HAT activity is responsible for opening the chromatin structure and increasing gene expression, whereas HDACs decrease acetylation levels, hence inducing a condensed chromatin state and limited transcriptional activity [23]. These mechanisms play a relevant role in cancer stem cells, so that acetylation and deacetylation can be considered key regulating processes influencing the expression of genes involved in viability, proliferation, stemness, and differentiation. Accordingly, treatments that enhance HAT activity or inhibits HDAC function have become a promising strategy to target cancer stem cells [24]. Diverse HDAC inhibitors have been successfully tested as anticancer compounds in experimental brain cancers [25, 26] (Table 1).

Given that HDACs regulate stemness features, studies have investigated its role in cancer stem cells. Parthenolide, a Histone Deacetylase 1 (HDAC1) inhibitor, was able to decrease *CD133* expression in MB cells. In addition, HDAC inhibitors trichostatin A (TSA) and tacedinaline induced significant decreases in *CD133* expression in Daoy and D283 Med MB cells, respectively. Sphere survival assay has shown that parthenolide and vorinostat (also known as SAHA) can decrease the viability of MB stem cell-enriched spheres [27].

Another HDAC inhibitor, sodium butyrate (NaB), was shown to reduce sphere formation and likely promote neuronal differentiation indicated by morphological changes and upregulation of Glutamate Ionotropic Receptor AMPA Type Subunit 2 (GRIA2) in MB cells [25]. NaB also reduced the expression of stemness genes BMI1 and CD133 at both transcriptional and protein content levels, while increasing global acetylation, in human MB cells [26]. In NB cells, the combination of retinoic acid and NaB was able to upregulate neuronal markers RNA Binding Fox-1 Homolog 3 (RBFOX3) (also known as NeuN) and Tubulin Beta 3 Class III (TUBB3) and downregulate BMI1. These results suggest that the combination of retinoids with epigenetic modulators might be an efficient novel strategy to inhibit NB tumor growth [28].

Valproic acid (VPA), a well-tolerated antiepileptic, has been identified as an HDAC inhibitor [29]. NB treatment with VPA was shown to increase cell death and improve phenotypic changes associated with differentiation, such

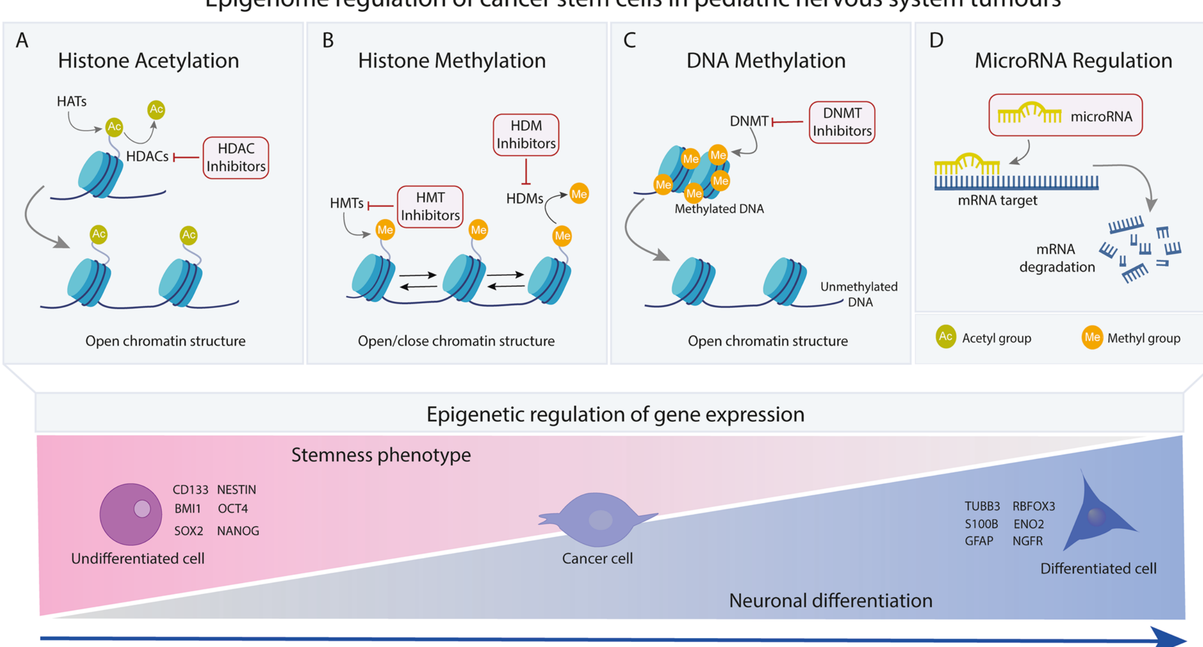

Fig. 1 Overview of main epigenetic modifications in pediatric nervous system tumors. Chromosomal DNA is packaged around histone octamer to form nucleosomes. Posttranslational histone modifications and DNA methylation act together to regulate the chromatin state. **A**-**B** Acetylation and methylation of histone tails are mediated by histone acetyl-transferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), and histone demethylases (HDMs). **C** DNA methylation is regulated by DNA-methyltransferase (DNMT). Inhibitors of main epigenetic factors can alter the epigenetic landscape and gene expression. **D** Epigenetic regulation can also occur by the action of non-coding RNAs (ncRNAs), such as microRNAs, that act as transcriptional regulators. All these epigenetic modifications interact with each other and are responsible for regulating the chromatin state, and consequently the expression of genes related to stemness phenotype and neuronal differentiation of pediatric nervous system tumors

as neurite extension and upregulation of neuronal markers Neuropeptide Y (*NPY)* and Growth Associated Protein 43 (*GAP43)* [30]. However, under certain conditions VPA can also induce CD133 expression and decrease sensitivity to cytostatic agents in NB cells [31]. Thereby, HDAC inhibitors may have limited efficacy as single agents. The combination of VPA and retinoic acid was more efficient than VPA alone in inducing differentiation indicated by increased neurite extension and upregulation of the differentiation marker Neurofilament Medium Chain (NEFM) in NB [32].

In chemoresistant NB cells, HDAC inhibition by vorinostat restored sensitivity to chemotherapy and reduced sphere forming ability. In addition, stemness genes, *SOX2, Insulin Like Growth Factor Binding Protein 3* (IGFBP3*)* and *Vimentin* (VIM*)* were downregulated in the presence of vorinostat [33]. MS-275, also known as Entinostat, is an HDAC inhibitor that preferentially inhibits class I HDACs. In NB cells, MS-275 was able to reduce growth, increase histone H3 and H4 acetylation, as well as increase expression of neurofilament and neuronal markers S100 Calcium Binding Protein B (S100B) and Glial Fibrillary Acidic Protein (GFAP) [34]. Moreover, the combination of MS-275 and acetazolamide, a pan carbonic anhydrase inhibitor, was capable to reduce the NB stem cell population through downregulating stemness genes POU Class 5 Homeobox 1 (POU5F1) (also known as OCT4), SOX2 and NANOG [35]. Another HDAC inhibitor that seems to have a relevant effect in NB cells is HKI 46F08. Treatment with HKI 46F08 was able to decrease clonogenic growth, cause morphological changes similar to neurite-like extensions and upregulate differentiation markers such as *NEFM* and *Microtubule Associated Protein 2* (MAP2*)* [36].

Another strategy to increase histone acetylation is the inhibition of specific classes of HDACs. Oehme et al. [37] show that Histone Deacetylase 8 (HDAC8) knockdown or selective inhibition was able to decrease proliferation and clone formation, induce differentiation, through the upregulation of differentiation markers *MAP2*, *NEFM*, *Neurotrophic Receptor Tyrosine Kinase 1* (NTRK1*)*, *TUBB3*, *GAP43*, and downregulation of stemness gene, *Nestin* (NES*)* in NB cells. When combining HDAC8 inhibition with retinoic acid agent, an increase in neurite outgrowths and expression of NEFM and NTRK1 in NB cells was exhibited. Also, in vivo results demonstrate that the combination of both agents is more efficient than either treatment alone

Table 1 Histone acetylation inhibitors associated with pediatric nervous system tumor stem cells

Compound	Target	Results	Clinical trials
Parthenolide	HDAC1	Downregulates *CD133* in MB cells and decreases sphere viability [27]	
Trichostatin A	HDAC class I/II	Downregulates *CD133* expression in Daoy [27]	
Tacedinaline	HDAC class I	Downregulates *CD133* expression in D283 med [27]	
Vorinostat	HDAC class I/II/IV	Decreases MB spheres viability [27]	Phase I [111, 112, 122–129]
		Downregulates stemness genes, reduces sphere forming ability and sensitizes NB resistant cells to chemotherapy [33]	Phase I/II [130–132] Phase II [133-134]
Sodium butyrate	HDAC class I/IIa	Reduces MB spheres formation, upregulates *Gria2* and downregulates stemness genes BMI1 and CD133 [25, 26]	
		Combination with retinoic acid upregulates RBFOX3 and TUBB3 and downregulates BMI1 in NB cells [28]	
Valproic acid	HDAC class I/II	Promotes neurite extension and upregulates *NPY* and *GAP43* in NB cells [30] Combination with temozolomide downregulates stem cells marker *musashi1* in pediatric glioma [43]	Phase I [107, 135–137] Phase II [138, 139] Phase III [140]
MS-275	HDAC class I	Reduces NB cell growth, upregulates S100beta and GFAP [34]	Phase I [141]
		Combination with acetazolamide in NB cells downregulates OCT4, SOX2 and NANOG [35]	Phase I/II [142]
HKI 46F08		Promotes neurite like extensions and upregulates *NEFM*, *SYN* and *MAP2* in NB cells [36]	
Selective inhibitor for HDAC8	HDAC8	Decreases NB proliferation and upregulates differentiation markers such as *MAP2* and *TUBB3* and downregulates *NES* [37]	
		Combination with retinoic acid increases neurite outgrowths and upregulates NEFM and NTRK1 expression [38]	
Selective inhibitor for HDAC1/2	HDAC1 and HDAC2	Decreases NB viability and induces differentiation. Combination with retinoic acid causes greater differentiation [39]	
Corin	HDACs and LSD1	Decreases NB growth in vitro and in vivo and regulates differentiation and stemness genes [40]	
Panobinostat	HDAC class I/ II/IV	Reduces DIPG tumor growth in vivo [42]	Phase I [116, 128, 143–146]
		Combination with inhibitor of AXL downregulates SOX2 and NES and prolongs survival of mice with DIPG xenografts [41]	
Depsipeptide	HDAC class I	Decreases expression of PCR2 components, including EZH2 and SUZ12, and upregulates CASZ1 in NB cells [50]	

in decreasing tumor proliferation, indicating a synergistic effect [38]. Moreover, HDAC1 and Histone Deacetylase 2 (HDAC2) inhibition in NB cells, through knockdown or pharmacological inhibition, was able to decrease viability and induce differentiation. Combination of HDAC1/2 inhibition and retinoic acid caused significantly greater differentiation and had a synergistic effect on NB viability [39].

DIPGs frequently have histone 3 lysine 27 mutations (H3K27M) resulting in epigenetic dysfunction. Thereby, epigenetic modifiers have shown great potential in the treatment of DIPGs. Anastas et al. [40] demonstrated that the use of Corin, a bifunctional inhibitor of HDACs and lysine-specific histone demethylase 1 (LSD1), was capable of decreasing growth in vitro and in vivo through increasing histone acetylation and methylation and regulating genes associated with neuronal differentiation and progenitor markers. Combining HDACi, panobinostat, with the inhibition of AXL, a receptor tyrosine kinase that is enrolled as a regulator of the mesenchymal transition, also shows meaningful results against DIPGs. Spheres treated with panobinostat or in combination with AXL inhibitor resulted in downregulation of stemness markers SOX2 and NES, and prolonged survival of mice with DIPG xenografts [41]. Grasso et al. [42] also demonstrated that panobinostat reduced tumor growth

in vivo models using DIPGs spheres. Moreover, VPA also shown potential in the treatment of pediatric glioma. Treatment with VPA alone or in combination with temozolomide (TZM) decreased cellular viability and downregulated stem cells marker *musashi1* in pediatric glioma cell lines [43].

Histone methylation

Histone methylation occurs predominantly on lysine (K) and arginine (R) residues and can happen at three different levels: mono-, di-, and tri-methylation. Histone methylation can also be associated with gene expression regulation. Recent discoveries show that methylation on specific residues can result in gene activation or repression. Gene activation has been associated with histone 4 lysine 20 methylation (H4K20me), histone 2B lysine 5 methylation (H2BK5me), histone 3 lysine 4 trimethylation (H3K4me3), histone 3 lysine 36 trimethylation (H3K36me3) and histone 3 lysine 79 trimethylation (H3K79me3), while histone 3 lysine 9 dimethylation (H3K9me2), histone 3 lysine 9 trimethylation (H3K9me3) and histone 3 lysine 27 trimethylation (H3K27me3) are usually related to gene repression [44]. The balance of methylation levels is sustained by histone methyltransferases (HMT) and histone demethylases (HDM). It is known that aberrant histone methylations can effect regulation of gene expression, differentiation and DNA damage repair, but recently it was established that histone methylation can also contribute to the acquisition of self-renewal features and cancer stem cell formation [45] (Table 2).

Enhancer of zeste homologue 2 (EZH2) is an HMT that participates on the Polycomb repressive complex 2 (PRC2), which catalyzes the trimethylation of histone 3 lysine 27 (H3K27). Several studies have shown that EZH2 can contribute to the origin of cells with cancer stem cell features in pancreatic, breast and brain tumors [46]. Moreover, inhibition of EZH2 affects cancer stem cells formation in MB cells demonstrating the importance of this methyltransferase on stemness of pediatric tumors [47, 48]. Furthermore, recent discoveries demonstrated that the protein maternal embryonic leucine-zipper kinase (MELK) collaborates with EZH2 in MB stem cells, suggesting that the two proteins act together to maintain cancer stem cell features [49].

In NB cells, upregulation of EZH2 was associated with maintenance of the undifferentiated state through regulation of tumor suppressor genes. Wang et al. [50] demonstrated that inhibition of EZH2 decreased cell growth, increased cells with neurite-like features and the expression of tumor suppressor genes such as Castor Zinc Finger 1 (*CASZ1)*, Clusterin (*CLU)*, Nerve Growth Factor Receptor (*NGFR)* and RUNX Family Transcription Factor 3 (*RUNX3)*. Furthermore, treatment with HDAC inhibitor, depsipeptide, was shown to decrease the expression of PRC2 components, including EZH2, and upregulate *CASZ1*, suggesting that PRC2 components have a relevant role on the undifferentiated state of NB tumors.

Lysine-specific demethylase 1 (LSD1) is a histone demethylase involved in the regulation of gene expression. Schulte, J and colleagues [51] demonstrated that knockdown or pharmacological inhibition of LSD1 decreased cellular growth of NB cells. Besides that, differentiated NB cells (with retinoic acid) showed lower expression of LSD1, suggesting the participation of LSD1 on cellular differentiation process. Histone methylation can also modulate MYC signaling, which is an important genetic mark in NB cells. Lysine Demethylase 4B (KDM4B) inhibition was able to regulate NB cell proliferation and differentiation in vitro and in vivo. Knockdown of KDM4B decreased the expression of *SOX2* and increased differentiation markers [52]. Another histone lysine demethylase relevant in NB is Lysine Demethylase

Table 2 Histone and DNA methylation inhibitors related with pediatric nervous system tumor stem cells

Compound	Target	Results	Clinical trials
EZH2 inhibitor	EZH2	Decreases proliferation MB stem cell formation and downregulates stemness markers in vitro and in vivo [47, 48]	Phase II [147, 148]
		*D*ecreases NB growth, improves neurite-like feature and upregulates tumor suppressor genes *CASZ1, CLU, NGFR* and *RUNX3* [50]	
GSK-J4	H3 methylation levels	Inhibits NB growth in vitro and in vivo, and upregulates differentiation markers such as *ENO2*, *CHD5*, *NGF* and *NRG1* [54]	
		In pediatric glioma GSK-J4 treatment increases K27me2 and K27me3 in cells with and without K27M mutation [56]	
Monoamine oxidase inhibitor	LSD1	Decreases NB growth and is involved in the differentiation process [51]	
DNA hypomethylating agents	Global DNA methylation	Increases CD133 expression in NB cell lines, suggesting that DNA methylation can be responsible for the regulation of CD133 expression [64]	Phase I [149–152] Phase I/Ib [117]
Nucleoside DNMT inhibitors	DNMT	Induces stem cell adhesion and upregulates TUBB3 [63]	

5B (KDM5B). KDM5B expression was associated with stem cell features in NB cells. Silencing KDM5B decreased cell invasion capability, sphere formation and was able to sensitize NB cells to cisplatin treatment [53]. Studies have shown that treatment with the histone demethylase inhibitor GSK-J4 is able to regulate H3 methylation levels, specially H3K27. In NB tumors, GSK-J4 inhibited NB growth in vitro and in vivo, and upregulated differentiation markers such as *Enolase 2 (ENO2)*, Nerve Growth Factor (*NGF)* and Neuregulin 1 (*NRG1)*. Also, combination with retinoic acid was able to overcome drug resistance [54].

In pediatric gliomas, mutations on the H3F3A gene, which encodes histone 3 variant H3.3, causes a substitution of lysine 27 for methionine (K27M) or glycine 34 to arginine (G34R), altering important sites of regulatory methylation [55]. Hashizume et al. [56] demonstrated that GSK-J4 treatment increased H3K27me2 and H3K27me3 in cells with and without K27M mutation. Despite that, GSK-J4 treatment seems to have different in vitro effects depending on K27 mutation status. Cells harboring K27M were more sensitive to GSK-J4 showing complete inhibition of clonogenic capabilities, while GSK-J4 had no effect on wild-type cells, suggesting that demethylation is a possible target for NB and pediatric glioma treatment. Moreover, pediatric gliomas with H3K27M mutation appear to affect EZH2 subunit and consequently reduce PRC2 activity [57, 58]. Mohammad et al. [59] showed that EZH2 inhibitors were able to reduce growth of H3K27M DIPG cells but had no effect on patient-derived primary glioma neural stem cell lines. Although histone methylation inhibition affects MB and NB stemness pathway, this epigenetic mechanism is not yet elucidated in pediatric gliomas.

DNA methylation

DNA methylation also has a significant role in cancer stem cells. Recent discoveries show a link between the undifferentiated phenotype and DNA methyltransferases [60]. DNA methyltransferases (DNMTs) are enzymes responsible for adding a methyl group from S-adenosyl methionine (SAM) to cytosine bases of CpG dinucleotides at gene promoters and regulatory regions, turning these regions less accessible for transcription. Thus, DNA hypermethylation can contribute to reduce gene expression; hence, recent reports show that an increase of methylation is common at promoters of tumor suppressor and differentiation genes, suggesting that DNA methylation is a relevant process in cancer stem cells [61].

In the past decade, research on the effect of non-nucleoside DNA methyltransferases inhibitor (DNMTi) in cancer has been increased [62]. Valente et al. [63] demonstrated that non-nucleoside DNMTi was able to induce stem cell adhesion and upregulate differentiation marker, *TUBB3,* in a MB stem cell model. These results suggest that this DNMTi is a potential treatment against MB stem cells.

Cancer stem cells are significantly enriched within CD133 + populations derived from all types of solid tumors, and this may raise the question if methylation status can regulate CD133 expression. Castresana and colleagues [64] have shown that treatment with the de-methylation agent 5-Azacytidine (5-AZA) increased CD133 expression at ribonucleic acid (RNA) and protein level in NB cell lines, suggesting that epigenetic changes can be responsible for the regulation of CD133 expression. Although the use of epigenetic modifiers have great potential against cancer stem cells, there are studies showing that short-term treatment with DNA methylation agents and/or histone deacetylase inhibitors can increase the expression of stemness genes in NB cells [65].

In pediatric gliomas, there is a shortcoming of studies exploring DNA methylation as a therapeutic target or its involvement on the stemness pathway, even though DNA hypomethylation has been considered a main driver for these tumors [58]. Recent findings in the DNA methylation profile indicate that pediatric HGG and DIPGs have different molecular subgroups with distinct epigenetic contributions [66]. The molecular signatures in pediatric gliomas brought to light specific oncogenic drivers and methylation patterns that impact clinical outcomes [67, 68].

Chromatin remodeling

Although histone modification and DNA methylation are major players in the epigenetic machinery, chromatin modifiers also have a relevant role in the chromatin integrity and accessibility. Chromatin remodelers are responsible for packaging the DNA and incorporating or releasing histones into the nucleosomes [69].

Recent discoveries demonstrated that the chromatin remodeler Lymphoid Specific (HELLS) has a significant role in MB tumors. Robinson et al. [70] showed that HELLS is especially important in the Sonic hedgehog (SHH) MB group, and the SHH pathway is capable of modulating HELLS expression. Also, in an embryonic stem cell model, researchers have shown that HELLS was capable of regulating stemness, suggesting that this chromatin remodeler can have a relevant role in cancer stem cell differentiation [71].

In NB tumors, elevated expression of the histone chaperone and epigenetic regulator, Chromatin Assembly Factor 1 Subunit A (CHAF1A), can be linked to an undifferentiated state of cancer cells. Silencing of CHAF1A caused morphologic changes associated with cell differentiation and increased expression levels of the neuronal marker TUBB3. Also, gene expression profiling revealed that CHAF1A

silencing was correlated with the repression of oncogenic pathways such as KRAS Proto-Oncogene, GTPase (KRAS), ALK Receptor Tyrosine Kinase (ALK), AKT Serine/Threonine Kinase 1 (AKT), and BMI1, suggesting that CHAF1A can prevent the differentiation process in NB tumors [72]. The plant homeodomain finger-containing protein 20 (PHF20) is considered an important epigenetic regulator, since it is a main reader of methylated states of histone H3 lysine 4 (H3K4) and participates in the lysine acetyltransferase complex MOF, that is capable of adding a acetyl group on the histone H4 lysine 16 (H4K16) [73]. PHF20 interacts with poly (ADP-ribose) polymerase 1 (PARP1) and directly binds to the promoter regions of stemness genes, such as *OCT4* and *SOX2*. Therefore, deletion of PHF20 was able to decrease NB cell proliferation, sphere formation, and increase differentiation features. These data suggest that PHF20 is involved in NB aggressiveness through the regulation of stemness genes [74].

Another chromatin remodeler associated with the maintenance of an undifferentiation state is the nucleosome-remodeling factor, Bromodomain PHD Finger Transcription Factor (BPTF). In pediatric HGG, BPTF appears to be a key regulator of tumor growth and differentiation. Green et al. [75] showed that silencing BPTF decreased SRY-Box Transcription Factor 10 (*SOX10)* and *GFAP* expression, and increased *TUBB3* and Oligodendrocyte Transcription Factor 3 (*OLIG3),* thus promoting differentiation of HGG cells. BPTF inhibition was able to reduce sphere growth and size, demonstrating a relevant role of BPTF in pediatric glioma stemness.

MicroRNAs

Recent findings suggest that microRNAs, which are a class of small non-coding RNA, are key regulators in human cancer. MicroRNAs commonly regulate gene expression by interacting with the 3'-untranslated region of specific mRNA targets leading to the degradation of RNA transcripts and ultimately to translational repression [76]. MicroRNAs can be down or upregulated in human tumors compared to normal tissues, but recent discoveries show that microRNA can also contribute to the formation and maintenance of cancer stem cells [77] (Table 3).

Venkataraman et al. [78] demonstrated that several microRNAs, in specific miR-128a, involved on the normal neuronal differentiation process appear to be downregulated in MB cells. Re-expression of miR-128a impaired MB cell growth and downregulated BMI1 expression and additionally reduced colony formation and tumor sphere size [79].

Patient data set in association with survival outcome is a practical tool to find new molecules relevant to cancer progression. For instance, upregulation of miR-199b-5p was associated with better prognosis in MB patients. Furthermore, overexpression of miR-199b-5p decreased MB proliferation and clonogenic capabilities, while positively regulating the differentiation marker *GFAP*. In vivo data also demonstrated that miR199b-5p expression was able to reduce tumor growth by reducing CD133+and CD15+populations. Also, de-methylation with 5-AZA was shown to upregulate miR-199b-5p expression in three MB cell lines, suggesting that 5-AZA may be a potential treatment for modulation of this microRNA [80, 81]. Another miRNA that is capable of reducing CD133+and CD15+population is miR-34a. This microRNA targets Delta-like 1 (Dll1) that is considered a regulator of the Notch pathway. mir-34a was capable to decrease Dll1 expression and affect cell proliferation and neuronal differentiation in MB cells [82].

In a MB cancer stem cell model, results indicate that miR-135a could be a potential tumor suppressor miRNA. miR-135a appears to be downregulated in highly tumorigenic cancer stem cells and its re-expression was able to impair the tumorigenesis process [83]. In addition, a model using MB stem cells versus RA-differentiated stem cells found that miR-135b, miR-195 and miR-145 seems to regulate proliferation, stemness maintenance and tumor invasiveness [84]. So far, we reveal that miRNA could potentially contribute to reduce tumorigenic potential. However, miRNAs might also be involved in cancer progression. Kaid et al. [85] found that miR-367 overexpression was able to enhance cell proliferation, invasion, and capability to form spheres, demonstrating that miR-367 has a pro-oncogenic activity in MB cells.

NB tumors are considered phenotypically heterogeneous, each having distinct differentiation and tumorigenic properties. The neuroblastic and non-neuronal phenotypes are examples of this heterogeneity. Samaraweera et al. [86] has reported that specific miRNAs define each phenotype. Upregulation of miR-21, miR-221 and miR-335 can be correlated with the non-neuronal phenotype, while miR-124 and miR-375 are specific to neuroblastic cells. Decreased expression of miR-335 in non-neuronal cells was able to regulate neuronal differentiation markers, Heart And Neural Crest Derivatives Expressed 1 (*HAND1)* and Jagged Canonical Notch Ligand 1 (*JAG1)*. Moreover, miR-124 overexpression is capable to induce neuronal differentiation in stem cells.

Recent discoveries have shown that microRNAs have important roles in cellular differentiation processes and that retinoic acid models has become an important tool for the detection of differential expression of microRNAs. Foley et al. [87] demonstrated that miR-10a and miR-10b were upregulated after retinoic acid treatment in NB cell lines. These data suggest that overexpression of this microRNAs can lead to a differentiated phenotype. Beveridge et al. [88] also showed that all members of the miR-17 cluster were downregulated after retinoic induced differentiation in NB cells. Screening approaches can be

Table 3 MicroRNA regulation associated with pediatric nervous system tumor stem cells

MicroRNA	Expression	Results
miR-128a	Downregulated	Re-expression decreases MB cell growth and downregulates BMI1 expression [78]
miR-218	Downregulated	Re-expression reduces MB growth, colony formation and sphere size [79]
miR-199b-5p	Downregulated	Over-expression upregulates *GFAP* and reduces tumor growth through downregulation of CD133 + and CD15 + population in vivo [80]
		Treatment with 5-aza upregulates miR-199b-5p in MB cell lines, suggesting that 5-AZA modulates this microRNA [81]
miR-34a	Downregulated	Targets Dll1, therefore affecting cell proliferation and neural differentiation in MB cells [82]
miR-135a	Downregulated	Low expression is associated with highly tumorigenic cancer stem cells features and re-expression impairs tumorigenesis in MB [83]
miR-135b	Downregulated	Upregulation is associated with MB stem cells differentiation [84]
miR-195	Upregulated	Downregulation is associated with MB stem cells differentiation [84]
miR-145		
miR-367	Upregulated	Over-expression enhances cell proliferation, invasion, and MB sphere formation [85]
miR-124	Downregulated	Overexpression induces differentiation in stem cells in NB [86]
miR-10a, miR-10b	Downregulated	Overexpression is associated with a differentiated phenotype in NB [87]
miR-17 cluster	Upregulated	Treatment with retinoic acid causes downregulation of all members miR-17 cluster in NB cells [88]
miR-124-3p, miR-135b-5p, miR-506-3p, miR-34a-5p miR-103a-3p	Downregulated	Re-expression increases differentiation markers GAP43, NSE and TUBB3, and reduces NB cell growth [89]
miR-432	Downregulated	Re-expression increases *CNR1, MAP2, BDNF, RARa* and *TH* expression, and downregulates *NESTIN* [90]
miR-449a	Downregulated	Overexpression decreases NB growth and upregulates TUBB3, NSE and GAP43 [91]
miR-7	Upregulated	Downregulation contributes to neurite outgrowth [92]
miR-214	Downregulated	Upregulation contributes to neurite outgrowth [92]
miR-340	Downregulated	Associated with aggressive NB tumors
		Upregulation decreases *SOX2* expression in response to retinoic acid treatment [93]
miR-137	Downregulated	Upregulation reduces PCR1/2 proteins such as EZH2 SUZ12, RING1B and BMI1 [94]
miR-25	Downregulated	Participates in SLC34A2-miR-25-Gsk3b signaling pathway to promote NB stemness [96]
miR-487	Downregulated	Overexpression reduces colony formation and downregulates *CD133* and *NES* in pediatric gliomas [100]
		Associated with prognosis in neuroblastoma [103]

considered a useful tool to find new targets. Zhao et al. [89] found 14 microRNAs involved in NB differentiation, and re-expression of miR-124-3p, miR-135b-5p, miR-506-3p, miR-34a-5p miR-103a-3p were capable to increase differentiation markers GAP43, ENO2 and TUBB3, and reduce NB cell growth.

Studies have found that the overexpression of miR-432 was able to increase the expression of neuronal marker, such as *MAP2*, Brain Derived Neurotrophic Factor (*BDNF)* and Retinoic Acid Receptor Alpha (*RARa)* in NB cells. Moreover, miR-432 also downregulated the stemness gene, NES [90]. Similarly, overexpression of miR-449a was able to decrease cellular growth and survival in NB cells, and to increase differentiation markers such as TUBB3, ENO2 and GAP43 [91]. In addition, Chen et al. [92] have shown that downregulation of miR-7 and upregulation of miR-214 can contribute to neurite outgrowth, demonstrating that these microRNAs were modulated during the differentiation of NB cells.

Epigenetic regulation by miRNAs has been associated with patient survival in NB tumors, suggesting that microRNAs could be prognostically relevant. Das et al. [93] have reported the association between DNA methylation, miRNA regulation and overall patient survival. miR-340 has been identified as an epigenetically silenced miRNA in aggressive NB tumors. Increased miR-340 expression has been found following 5-AZA and retinoic acid treatment, showing that this microRNA can be modulated by DNA methylation and associated with differentiation. Moreover, the authors have discovered that upregulation of miR-340 is capable of decreasing *SOX2* expression in response to retinoic acid treatment.

As it was mentioned above, an important epigenetic mechanism in several tumors is the activity of histone methyltransferase EZH2. Ren et al. demonstrated that this

enzyme can be regulated by miR-137 in NB cells. A model using resveratrol showed that upregulation of miR-137 was capable of reducing EZH2 level. This treatment can also reduce other PRC2 and Polycomb repressive complex 1 (PCR1) proteins, such as Embryonic Ectoderm Development (EED), SUZ12, and BMI1, suggesting that miR-137 was able to modulate relevant epigenetics proteins [94]. Likewise, microRNAs can be modulated by different factors, an example is the let-7 family regulation by the protein Lin-28 Homolog B (LIN28B). LIN28B was able to repress let-7 miRNAs and upregulate MYCN Proto-Oncogene and BHLH Transcription Factor (MYCN) protein expression in NB cells. These alterations result in impairment of the differentiation process in NB cells and normal neuroblasts [95]. Chen et al. [96] have found that the regulation of stemness genes Aldehyde Dehydrogenase 1 Family Member A1 (ALDH1A1) and NANOG was associated with Solute Carrier Family 34 Member 2 (SLC34A2) expression. The transcription factor SLC34A2 directly binds to the promoter of miR-25, enhancing its expression in NB cells and spheres. Hence, miR-25 is capable to bind with Glycogen Synthase Kinase 3 Beta (Gsk3b), activating the Wnt signaling pathway. Therefore, SLC34A2 promotes NB stemness through miR-25-Gsk3b regulation.

In cerebellar neural stem cells (NSC), the Hedgehog-Gli pathway is essential for NANOG expression and maintenance of self-renewal features. In a model using differentiated NSC vs normal NSC, it has been shown that the protein Forkhead Box M1 (Foxm1), which is regulated by GLI Family Zinc Finger (Gli) and NANOG, was able to modulate miR-130b, miR-301a, and members of the miR-15–16 and miR-17–92 clusters. Moreover, inhibition of miR-130b, miR-301a, and miR-19a was able to reduce sphere formation and upregulate differentiation markers [97]. Since the Hedgehog pathway is a relevant pathway in certain types of MB [98] and NB tumors [99], these findings could indicate similar mechanisms for these two pediatric tumors.

In pediatric LGG and glioneuronal tumors, Ames et al. [100] demonstrated that 61 microRNAs are differentially expressed in tumors compared to brain tissue. Furthermore, overexpression of miR-487b in a pediatric glioma cell line was able to reduce colony formation and decrease *CD133* and *NES* expression, suggesting that microRNA have a pivotal role in regulating stemness in pediatric glioma. Likewise, the 14q32 cluster seems to be relevant to high grade gliomas. Jha et al. [101] have shown that several 14q32 microRNAs, including miR-431, miR-433, miR-380, miR-323a, miR-329, miR-543, miR-1185, miR-487b, miR-539, miR-487a, miR- 485, miR-668, miR-154 and miR-410 are downregulated in pediatric gliomas. Also, underexpression of 14q32 miRNAs has been reported in MB [102] and some microRNAs of the 14q32 cluster, especially miR-487b and miR-410, have been associated with prognosis in NB [103].

Conclusions and perspectives

In this review, we have summarized and highlighted epigenetic modulators as promising drug targets for cancer stem cells in pediatric nervous system tumors. Despite that, most of these epigenetic modifications are still far from clinical trials and approved drug use. Mostly, due to difficulties in modulating these specific epigenetic regulators, only six epigenetic drugs are approved by the Food and Drug Administration (FDA): two DNMT inhibitors and four HDAC inhibitors, mainly for myelodysplastic syndrome (MDS), cutaneous T-cell lymphoma (CTCL), and peripheral T-cell lymphoma (PTCL) [104]. DNMT inhibitors Azacitidine and Decitabine, approved for patients with MDS, improved overall survival compared to conventional care regimens or supportive care [152, 153]. Moreover, HDAC inhibitors, Vorinostat, and Depsipeptide, authorized for the treatment of CTCL patients, showed clinical benefits with objective or complete responses in phase II trials [154, 155]. Belinostat, approved for PTCL patients, showed an overall response rate in almost 30% of cases, including complete and partial responses in phase II trials [156]. Furthermore, a phase II trial showed that inclusion of Panobinostat to Bortezomib regimen has clinical activity and benefits to relapsed or/and refractory multiple myeloma patients [157]. The main side effects associated with these epigenetic drugs were myelosuppression and gastrointestinal symptoms [154–156, 158–160].

Given that cancer stem cells can be associated with tumor recurrence and metastatic potential [105, 106], trials with relapsed or recurrent tumors using epigenetic modulators can produce relevant results in the cancer stem cells context. Clinical trials in pediatric patients with nervous system tumors seem to focus mostly on histone deacetylase inhibitors. Phase I trials with VPA show well tolerated doses in children with central nervous system tumors. In patients with HGG or DIPG, a phase II trial showed that the addition of VPA and bevacizumab to the radiation regimen was well tolerated [107, 108]. A previous trial also indicated that VPA in combination with radio and chemotherapy is well tolerated and have encouraging response rates [109]. In addition, there is currently an ongoing phase III trial with VPA plus TMZ for pediatric glioma patients [140]. Moreover, trials with Vorinostat also show promising responses. In a phase I and II trial five out of fifty-two patients achieved partial responses. Among these patients, three had HGG diagnosis, suggesting that Vorinostat can penetrate blood–brain barrier [110]. A phase I trial combining Vorinostat and TMZ in relapsed brain or spinal cord tumors had also showed promising results of stable disease or partial response [111]. For patients with relapsed or refractory NB, a phase I trial with vorinostat

as a radio sensitizing agent resulted in establishment of a tolerable dose of vorinostat with MIBG (131I-metaiodobenzylguanidine) [112]. Completed phase II trials with HDAC inhibitors indicate that adverse effects described for pediatric patients with nervous system tumors are similar to side effects of FDA-approved epigenetic drugs [108, 109].

Besides that, there are currently several trials active or in recruitment for patients with pediatric nervous system tumors. Phase I trial with HDACi, Entinostat, for recurrent solid tumors as single agent or in combination with an immunotherapy agent against PD-1 receptor, Nivolumab [113, 114]. For patients with HGG or DIPG there are also trials focusing on the combination of vorinostat, TZM or bevacizumab, and panobinostat with the proteasome inhibitor Marizomib, respectively [115, 116]. DNMTi are also being tested for patients with recurrent brain tumors using azacitidine [117]. These open trials illustrate the potential of epigenetic drugs for the treatment of pediatric patients and indicate promising alternative treatments.

The first and second wave of epigenetic drugs resulted in the development of the HDAC and DNMT inhibitors already approved by FDA [118]. In the last decade, the discovery of small-molecules capable of targeting specific epigenetic components led to the development of clinically relevant drugs that are in trials for several types of tumors. Phases I and II trial with EZH2 inhibitor, Tazemetostat, are active for pediatric patients with relapsed or refractory advanced solid tumors, and non-Hodgkin lymphomas [147, 148]. Completed phase I or II trial using Tazemetostat in refractory B-cell non-Hodgkin lymphoma indicated that inhibition of EZH2 has clinical benefits with complete and partial responses in these oncological patients [119]. Trials with methyltransferase inhibitors are also open for cancer patients [120]. Also, evaluation of microRNA-10b expression level is in trial for adult patients with glioma [121]. Depending on results, this and other epigenetic modulators will potentially have a role in the improvement of survival rates of patients with medulloblastoma, neuroblastoma and pediatric glioma in the future.

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References

1. Steliarova-Foucher E, Colombet M, Ries LAG et al (2017) International incidence of childhood cancer, 2001–10: a population-based registry study. Lancet Oncol 18:719–731. https://doi.org/10.1016/S1470-2045(17)30186-9
2. Ma X, Liu Y, Liu Y et al (2018) Pan-cancer genome and transcriptome analyses of 1,699 paediatric leukaemias and solid tumours. Nature 555:371–376. https://doi.org/10.1038/nature25795
3. Gröbner SN, Worst BC, Weischenfeldt J et al (2018) The landscape of genomic alterations across childhood cancers. Nature 555:321–327. https://doi.org/10.1038/nature25480
4. Downing JR, Wilson RK, Zhang J et al (2012) The pediatric cancer genome project. Nat Genet 44:619–622. https://doi.org/10.1038/ng.2287
5. Huether R, Dong L, Chen X et al (2014) The landscape of somatic mutations in epigenetic regulators across 1,000 paediatric cancer genomes. Nat Commun. https://doi.org/10.1038/ncomms4630
6. Siegel RL, Miller KD, Jemal A (2020) Cancer statistics, 2020. Cancer J Clin 70:7–30. https://doi.org/10.3322/caac.21590
7. Qureshi IA, Mehler MF (2011) Epigenetics, nervous system tumors, and cancer stem cells. Cancers 3:3525–3556. https://doi.org/10.3390/cancers3033525
8. Tomolonis JA, Agarwal S, Shohet JM (2017) Neuroblastoma pathogenesis: deregulation of embryonic neural crest development. Cell Tissue Res 372:245–262. https://doi.org/10.1007/s00441-017-2747-0
9. Lu QR, Qian L, Zhou X (2019) Developmental origins and oncogenic pathways in malignant brain tumors. Wiley Interdiscip Rev. https://doi.org/10.1002/wdev.342
10. Han JW, Yoon Y-S (2012) Epigenetic landscape of pluripotent stem cells. Antioxid Redox Signal 17:205–223. https://doi.org/10.1089/ars.2011.4375
11. Hemmati HD, Nakano I, Lazareff JA et al (2003) Cancerous stem cells can arise from pediatric brain tumors. Proc Natl Acad Sci 100:15178–15183. https://doi.org/10.1073/pnas.2036535100
12. Walton JD, Kattan DR, Thomas SK et al (2004) Characteristics of stem cells from human neuroblastoma cell lines and in tumors. Neoplasia 6:838–845. https://doi.org/10.1593/neo.04310

13. Thirant C, Bessette B, Varlet P et al (2011) Clinical relevance of tumor cells with stem-like properties in pediatric brain tumors. PLoS ONE. https://doi.org/10.1371/journal.pone.0016375
14. Northcott PA, Robinson GW, Kratz CP et al (2019) Medulloblastoma. Nat Rev Dis Primers. https://doi.org/10.1038/s41572-019-0063-6
15. Johnsen JI, Dyberg C, Wickström M (2019) Neuroblastoma—a neural crest derived embryonal malignancy. Front Mol Neurosci. https://doi.org/10.3389/fnmol.2019.00009
16. Funakoshi Y, Hata N, Kuga D et al (2021) Pediatric glioma: an update of diagnosis, biology, and treatment. Cancers 13:758. https://doi.org/10.3390/cancers13040758
17. Lawlor ER, Thiele CJ (2012) Epigenetic changes in pediatric solid tumors: promising new targets. Clin Cancer Res 18:2768–2779. https://doi.org/10.1158/1078-0432.CCR-11-1921
18. Franco SS, Szczesna K, Iliou MS et al (2016) In vitro models of cancer stem cells and clinical applications. BMC Cancer. https://doi.org/10.1186/s12885-016-2774-3
19. Singh SK, Hawkins C, Clarke ID et al (2004) Identification of human brain tumour initiating cells. Nature 432:396–401. https://doi.org/10.1038/nature03128
20. Ross RA, Walton JD, Han D et al (2015) A distinct gene expression signature characterizes human neuroblastoma cancer stem cells. Stem Cell Res 15:419–426. https://doi.org/10.1016/j.scr.2015.08.008
21. Pandian V, Ramraj S, Khan FH et al (2015) Metastatic neuroblastoma cancer stem cells exhibit flexible plasticity and adaptive stemness signaling. Stem Cell Res Ther. https://doi.org/10.1186/s13287-015-0002-8
22. Nightingale KP, O'Neill LP, Turner BM (2006) Histone modifications: signalling receptors and potential elements of a heritable epigenetic code. Curr Opin Genet Dev 16:125–136. https://doi.org/10.1016/j.gde.2006.02.015
23. Yang X-J, Seto E (2007) HATs and HDACs: from structure, function and regulation to novel strategies for therapy and prevention. Oncogene 26:5310–5318. https://doi.org/10.1038/sj.onc.1210599
24. Liu N, Li S, Wu N, Cho K-S (2017) Acetylation and deacetylation in cancer stem-like cells. Oncotarget 8:89315–89325. https://doi.org/10.18632/oncotarget.19167
25. Nör C, Sassi FA, Farias CBD et al (2013) The histone deacetylase inhibitor sodium butyrate promotes cell death and differentiation and reduces neurosphere formation in human medulloblastoma cells. Mol Neurobiol 48:533–543. https://doi.org/10.1007/s12035-013-8441-7
26. Jaeger MDC, Ghisleni EC, Cardoso PS et al (2020) HDAC and MAPK/ERK inhibitors cooperate to reduce viability and stemness in medulloblastoma. J Mol Neurosci 70:981–992. https://doi.org/10.1007/s12031-020-01505-y
27. Yuan J, Luceño NL, Sander B, Golas MM (2017) Synergistic anti-cancer effects of epigenetic drugs on medulloblastoma cells. Cell Oncol 40:263–279. https://doi.org/10.1007/s13402-017-0319-7
28. Almeida VR, Vieira IA, Buendia M et al (2016) Combined treatments with a retinoid receptor agonist and epigenetic modulators in human neuroblastoma cells. Mol Neurobiol 54:7610–7619. https://doi.org/10.1007/s12035-016-0250-3
29. Chateauvieux S, Morceau F, Dicato M, Diederich M (2010) Molecular and therapeutic potential and toxicity of valproic acid. J Biomed Biotechnol 2010:1–18. https://doi.org/10.1155/2010/479364
30. Stockhausen M-T, Sjölund J, Manetopoulos C, Axelson H (2005) Effects of the histone deacetylase inhibitor valproic acid on Notch signalling in human neuroblastoma cells. Br J Cancer 92:751–759. https://doi.org/10.1038/sj.bjc.6602309
31. Khalil MA, Hraběta J, Groh T et al (2016) Valproic acid increases CD133 positive cells that show low sensitivity to cytostatics in neuroblastoma. PLoS ONE. https://doi.org/10.1371/journal.pone.0162916
32. Hahn CK, Ross KN, Warrington IM et al (2008) Expression-based screening identifies the combination of histone deacetylase inhibitors and retinoids for neuroblastoma differentiation. Proc Natl Acad Sci 105:9751–9756. https://doi.org/10.1073/pnas.0710413105
33. Zheng X, Naiditch J, Czurylo M et al (2013) Differential effect of long-term drug selection with doxorubicin and vorinostat on neuroblastoma cells with cancer stem cell characteristics. Cell Death Dis. https://doi.org/10.1038/cddis.2013.264
34. Tsui, M. K. H (2009) Histone deacetylase inhibitor MS-275 inhibits neuroblastoma cell growth by inducing cell cycle arrest, apoptosis, differentiation and by targeting its tumor stem cell population. Thesis, University of Toronto
35. Mokhtari RB, Baluch N, Tsui MKH et al (2017) Acetazolamide potentiates the anti-tumor potential of HDACi, MS-275, in neuroblastoma. BMC Cancer. https://doi.org/10.1186/s12885-017-3126-7
36. Wegener D, Deubzer HE, Oehme I et al (2008) HKI 46F08, a novel potent histone deacetylase inhibitor, exhibits antitumoral activity against embryonic childhood cancer cells. Anticancer Drugs 19:849–857. https://doi.org/10.1097/cad.0b013e32830efbeb
37. Oehme I, Deubzer HE, Wegener D et al (2008) Histone deacetylase 8 in neuroblastoma tumorigenesis. Clin Cancer Res 15:91–99. https://doi.org/10.1158/1078-0432.CCR-08-0684
38. Rettig I, Koeneke E, Trippel F et al (2015) Selective inhibition of HDAC8 decreases neuroblastoma growth in vitro and in vivo and enhances retinoic acid-mediated differentiation. Cell Death Dis. https://doi.org/10.1038/cddis.2015.24
39. Frumm SM, Fan ZP, Ross KN et al (2013) Selective HDAC1/HDAC2 Inhibitors Induce neuroblastoma differentiation. Chem Biol 20:713–725. https://doi.org/10.1016/j.chembiol.2013.03.020
40. Anastas JN, Zee BM, Kalin JH et al (2019) Re-programing chromatin with a bifunctional LSD1/HDAC inhibitor induces therapeutic differentiation in DIPG. Cancer Cell. https://doi.org/10.1016/j.ccell.2019.09.005
41. Meel MH, Gooijer MCD, Metselaar DS et al (2020) Combined therapy of AXL and HDAC inhibition reverses mesenchymal transition in diffuse intrinsic pontine glioma. Clin Cancer Res 26:3319–3332. https://doi.org/10.1158/1078-0432.CCR-19-3538
42. Grasso CS, Tang Y, Truffaux N et al (2015) Functionally defined therapeutic targets in diffuse intrinsic pontine glioma. Nat Med 21:555–559. https://doi.org/10.1038/nm.3855
43. Pötschke R, Gielen G, Pietsch T et al (2019) Musashi1 enhances chemotherapy resistance of pediatric glioblastoma cells in vitro. Pediatr Res 87:669–676. https://doi.org/10.1038/s41390-019-0628-9
44. Barski A, Cuddapah S, Cui K et al (2007) High-resolution profiling of histone methylations in the human genome. Cell 129:823–837. https://doi.org/10.1016/j.cell.2007.05.009
45. Wainwright EN, Scaffidi P (2017) Epigenetics and cancer stem cells: unleashing, hijacking, and restricting cellular plasticity. Trends in Cancer 3:372–386. https://doi.org/10.1016/j.trecan.2017.04.004
46. Wen Y, Cai J, Hou Y et al (2017) Role of EZH2 in cancer stem cells: from biological insight to a therapeutic target. Oncotarget 8:37974–37990. https://doi.org/10.18632/oncotarget.16467
47. Miele E, Valente S, Alfano V et al (2017) The histone methyltransferase EZH2 as a druggable target in SHH medulloblastoma cancer stem cells. Oncotarget 8:68557–68570. https://doi.org/10.18632/oncotarget.19782

48. Alimova I, Venkataraman S, Harris P et al (2012) Targeting the enhancer of zeste homologue 2 in medulloblastoma. Int J Cancer 131:1800–1809. https://doi.org/10.1002/ijc.27455
49. Liu H, Sun Q, Sun Y et al (2017) MELK and EZH2 cooperate to regulate medulloblastoma cancer stem-like cell proliferation and differentiation. Mol Cancer Res 15:1275–1286. https://doi.org/10.1158/1541-7786.MCR-17-0105
50. Wang C, Liu Z, Woo C-W et al (2011) EZH2 mediates epigenetic silencing of neuroblastoma suppressor genes CASZ1, CLU, RUNX3, and NGFR. Can Res 72:315–324. https://doi.org/10.1158/0008-5472.CAN-11-0961
51. Schulte JH, Lim S, Schramm A et al (2009) Lysine-specific demethylase 1 is strongly expressed in poorly differentiated neuroblastoma: implications for therapy. Can Res 69:2065–2071. https://doi.org/10.1158/0008-5472.CAN-08-1735
52. Yang J, Altahan AM, Hu D et al (2015) The role of histone demethylase KDM4B in myc signaling in neuroblastoma. J Natl Cancer Inst. https://doi.org/10.1093/jnci/djv080
53. Kuo Y-T, Liu Y-L, Adebayo BO et al (2015) JARID1B expression plays a critical role in chemoresistance and stem cell-like phenotype of neuroblastoma cells. PLoS ONE. https://doi.org/10.1371/journal.pone.0125343
54. Lochmann TL, Powell KM, Ham J et al (2018) Targeted inhibition of histone H3K27 demethylation is effective in high-risk neuroblastoma. Sci Transl Med. https://doi.org/10.1126/scitranslmed.aao4680
55. Schwartzentruber J, Korshunov A, Liu X-Y et al (2012) Driver mutations in histone H3.3 and chromatin remodelling genes in paediatric glioblastoma. Nature 482:226–231. https://doi.org/10.1038/nature10833
56. Hashizume R, Andor N, Ihara Y et al (2014) Pharmacologic inhibition of histone demethylation as a therapy for pediatric brainstem glioma. Nat Med 20:1394–1396. https://doi.org/10.1038/nm.3716
57. Lewis PW, Muller MM, Koletsky MS et al (2013) Inhibition of PRC2 activity by a gain-of-function H3 mutation found in pediatric glioblastoma. Science 340:857–861. https://doi.org/10.1126/science.1232245
58. Bender S, Tang Y, Lindroth AM et al (2013) Reduced H3K27me3 and DNA hypomethylation are major drivers of gene expression in K27M mutant pediatric high-grade gliomas. Cancer Cell 24:660–672. https://doi.org/10.1016/j.ccr.2013.10.006
59. Mohammad F, Weissmann S, Leblanc B et al (2017) EZH2 is a potential therapeutic target for H3K27M-mutant pediatric gliomas. Nat Med 23:483–492. https://doi.org/10.1038/nm.4293
60. Jin B, Ernst J, Tiedemann RL et al (2012) Linking DNA methyltransferases to epigenetic marks and nucleosome structure genome-wide in human tumor cells. Cell Rep 2:1411–1424. https://doi.org/10.1016/j.celrep.2012.10.017
61. Pfeifer G (2018) Defining driver DNA methylation changes in human cancer. Int J Mol Sci 19:1166. https://doi.org/10.3390/ijms19041166
62. Pechalrieu D, Etievant C, Arimondo PB (2017) DNA methyltransferase inhibitors in cancer: from pharmacology to translational studies. Biochem Pharmacol 129:1–13. https://doi.org/10.1016/j.bcp.2016.12.004
63. Valente S, Liu Y, Schnekenburger M et al (2014) Selective non-nucleoside inhibitors of human DNA methyltransferases active in cancer including in cancer stem cells. J Med Chem 57:701–713. https://doi.org/10.1021/jm4012627
64. Castresana (2010) Analysis of stemness gene expression and CD133 abnormal methylation in neuroblastoma cell lines. Oncol Rep. https://doi.org/10.3892/or_00000993
65. Ikegaki N, Shimada H, Fox AM et al (2013) Transient treatment with epigenetic modifiers yields stable neuroblastoma stem cells resembling aggressive large-cell neuroblastomas. Proc Natl Acad Sci 110:6097–6102. https://doi.org/10.1073/pnas.1118262110
66. Mackay A, Burford A, Carvalho D et al (2017) Integrated molecular meta-analysis of 1,000 pediatric high-grade and diffuse intrinsic pontine glioma. Cancer Cell. https://doi.org/10.1016/j.ccell.2017.08.017
67. Korshunov A, Ryzhova M, Hovestadt V et al (2015) Integrated analysis of pediatric glioblastoma reveals a subset of biologically favorable tumors with associated molecular prognostic markers. Acta Neuropathologica 129:669–678. https://doi.org/10.1007/s00401-015-1405-4
68. Korshunov A, Schrimpf D, Ryzhova M et al (2017) H3-/IDH-wild type pediatric glioblastoma is comprised of molecularly and prognostically distinct subtypes with associated oncogenic drivers. Acta Neuropathol 134:507–516. https://doi.org/10.1007/s00401-017-1710-1
69. Nair SS, Kumar R (2012) Chromatin remodeling in cancer: a gateway to regulate gene transcription. Mol Oncol 6:611–619. https://doi.org/10.1016/j.molonc.2012.09.005
70. Robinson MH, Maximov V, Lallani S et al (2019) Upregulation of the chromatin remodeler HELLS is mediated by YAP1 in Sonic Hedgehog medulloblastoma. Sci Rep. https://doi.org/10.1038/s41598-019-50088-1
71. Xi S, Geiman TM, Briones V et al (2009) Lsh participates in DNA methylation and silencing of stem cell genes. Stem Cells 27:2691–2702. https://doi.org/10.1002/stem.183
72. Barbieri E, Preter KD, Capasso M et al (2013) Histone chaperone CHAF1A inhibits differentiation and promotes aggressive neuroblastoma. Can Res 74:765–774. https://doi.org/10.1158/0008-5472.CAN-13-1315
73. Klein BJ, Wang X, Cui G et al (2016) PHF20 readers link methylation of histone H3K4 and p53 with H4K16 acetylation. Cell Rep 17:1158–1170. https://doi.org/10.1016/j.celrep.2016.09.056
74. Long W, Zhao W, Ning B et al (2018) PHF20 collaborates with PARP1 to promote stemness and aggressiveness of neuroblastoma cells through activation of SOX2 and OCT4. J Mol Cell Biol 10:147–160. https://doi.org/10.1093/jmcb/mjy007
75. Green AL, Desisto J, Flannery P et al (2019) BPTF regulates growth of adult and pediatric high-grade glioma through the MYC pathway. Oncogene 39:2305–2327. https://doi.org/10.1038/s41388-019-1125-7
76. Lee S, Vasudevan S (2012) Post-transcriptional Stimulation of Gene Expression by MicroRNAs Advances in Experimental Medicine and Biology. Ten Years of Progress in GW/P Body Res. https://doi.org/10.1007/978-1-4614-5107-5_7
77. Garg N, Vijayakumar T, Bakhshinyan D et al (2015) MicroRNA regulation of brain tumour initiating cells in central nervous system tumours. Stem Cells Int 2015:1–15. https://doi.org/10.1155/2015/141793
78. Venkataraman S, Alimova I, Fan R et al (2010) MicroRNA 128a increases intracellular ROS level by targeting Bmi-1 and inhibits medulloblastoma cancer cell growth by promoting senescence. PLoS ONE. https://doi.org/10.1371/journal.pone.0010748
79. Venkataraman S, Birks DK, Balakrishnan I et al (2013) MicroRNA 218 acts as a tumor suppressor by targeting multiple cancer phenotype-associated genes in medulloblastoma. J Biol Chem 288:1918–1928. https://doi.org/10.1074/jbc.M112.396762
80. Garzia L, Andolfo I, Cusanelli E et al (2009) MicroRNA-199b-5p impairs cancer stem cells through negative regulation of HES1 in medulloblastoma. PLoS ONE. https://doi.org/10.1371/journal.pone.0004998
81. Andolfo I, Liguori L, Antonellis PD et al (2012) The micro-RNA 199b–5p regulatory circuit involves Hes1, CD15, and epigenetic modifications in medulloblastoma. Neuro Oncol 14:596–612. https://doi.org/10.1093/neuonc/nos002

82. Antonellis PD, Medaglia C, Cusanelli E et al (2011) MiR-34a targeting of notch ligand delta-Like 1 impairs CD15 /CD133 tumor-propagating cells and supports neural differentiation in medulloblastoma. PLoS ONE. https://doi.org/10.1371/journal.pone.0024584
83. Hemmesi K, Squadrito ML, Mestdagh P et al (2015) miR-135aInhibits cancer stem cell-driven medulloblastoma development by directly repressingArhgef6expression. Stem Cells 33:1377–1389. https://doi.org/10.1002/stem.1958
84. Catanzaro G, Besharat ZM, Garg N et al (2016) MicroRNAs-proteomic networks characterizing human medulloblastoma-SLCs. Stem Cells Int 2016:1–10. https://doi.org/10.1155/2016/2683042
85. Kaid C, Silva PBG, Cortez BA et al (2015) miR-367 promotes proliferation and stem-like traits in medulloblastoma cells. Cancer Sci 106:1188–1195. https://doi.org/10.1111/cas.12733
86. Samaraweera L, Grandinetti KB, Huang R et al (2014) MicroRNAs define distinct human neuroblastoma cell phenotypes and regulate their differentiation and tumorigenicity. BMC Cancer. https://doi.org/10.1186/1471-2407-14-309
87. Foley NH, Bray I, Watters KM et al (2011) MicroRNAs 10a and 10b are potent inducers of neuroblastoma cell differentiation through targeting of nuclear receptor corepressor 2. Cell Death Differ 18:1089–1098. https://doi.org/10.1038/cdd.2010.172
88. Beveridge NJ, Tooney PA, Carroll AP et al (2009) Down-regulation of miR-17 family expression in response to retinoic acid induced neuronal differentiation. Cell Signal 21:1837–1845. https://doi.org/10.1016/j.cellsig.2009.07.019
89. Zhao Z, Ma X, Hsiao T-H et al (2014) A high-content morphological screen identifies novel microRNAs that regulate neuroblastoma cell differentiation. Oncotarget 5:2499–2512. https://doi.org/10.18632/oncotarget.1703
90. Das E, Bhattacharyya NP (2014) MicroRNA-432 contributes to dopamine cocktail and retinoic acid induced differentiation of human neuroblastoma cells by targeting NESTIN and RCOR1 genes. FEBS Lett 588:1706–1714. https://doi.org/10.1016/j.febslet.2014.03.015
91. Zhao Z, Ma X, Sung D et al (2015) microRNA-449a functions as a tumor suppressor in neuroblastoma through inducing cell differentiation and cell cycle arrest. RNA Biol 12:538–554. https://doi.org/10.1080/15476286.2015.1023495
92. Chen H, Shalom-Feuerstein R, Riley J et al (2010) miR-7 and miR-214 are specifically expressed during neuroblastoma differentiation, cortical development and embryonic stem cells differentiation, and control neurite outgrowth in vitro. Biochem Biophys Res Commun 394:921–927. https://doi.org/10.1016/j.bbrc.2010.03.076
93. Das S, Bryan K, Buckley PG et al (2012) Modulation of neuroblastoma disease pathogenesis by an extensive network of epigenetically regulated microRNAs. Oncogene 32:2927–2936. https://doi.org/10.1038/onc.2012.311
94. Ren X, Bai X, Zhang X et al (2015) quantitative nuclear proteomics identifies that miR-137-mediated EZH2 Reduction Regulates Resveratrol-induced Apoptosis of Neuroblastoma cells*. Mol Cell Proteom 14:316–328. https://doi.org/10.1074/mcp.M114.041905
95. Molenaar JJ, Domingo-Fernández R, Ebus ME et al (2012) LIN28B induces neuroblastoma and enhances MYCN levels via let-7 suppression. Nat Genet 44:1199–1206. https://doi.org/10.1038/ng.2436
96. Chen J, Wang P, Cai R et al (2019) SLC34A2 promotes neuroblastoma cell stemness via enhancement of miR-25/Gsk3β-mediated activation of Wnt/β-catenin signaling. FEBS Open Bio 9:527–537. https://doi.org/10.1002/2211-5463.12594
97. Besharat ZM, Abballe L, Cicconardi F et al (2018) Foxm1 controls a pro-stemness microRNA network in neural stem cells. Sci Rep. https://doi.org/10.1038/s41598-018-21876-y
98. Huang S, Yang J-Y (2015) Targeting the hedgehog pathway in pediatric medulloblastoma. Cancers 7:2110–2123. https://doi.org/10.3390/cancers7040880
99. Wickström M, Dyberg C, Shimokawa T et al (2012) Targeting the hedgehog signal transduction pathway at the level of GLI inhibits neuroblastoma cell growthin vitroandin vivo. Int J Cancer 132:1516–1524. https://doi.org/10.1002/ijc.27820
100. Ames HM, Yuan M, Vizcaíno MA et al (2016) MicroRNA profiling of low-grade glial and glioneuronal tumors shows an independent role for cluster 14q32.31 member miR-487b. Mod Pathol 30:204–216. https://doi.org/10.1038/modpathol.2016.177
101. Jha P, Agrawal R, Pathak P et al (2015) Genome-wide small noncoding RNA profiling of pediatric high-grade gliomas reveals deregulation of several miRNAs, identifies downregulation of snoRNA cluster HBII-52 and delineates H3F3A and TP53 mutant-specific miRNAs and snoRNAs. Int J Cancer 137:2343–2353. https://doi.org/10.1002/ijc.29610
102. Lucon DR, Rocha CDS, Craveiro RB et al (2013) Downregulation of 14q32 microRNAs in primary human desmoplastic medulloblastoma. Front Oncol. https://doi.org/10.3389/fonc.2013.00254
103. Gattolliat C-H, Thomas L, Ciafrè SA et al (2011) Expression of miR-487b and miR-410 encoded by 14q32.31 locus is a prognostic marker in neuroblastoma. Br J Cancer 105:1352–1361. https://doi.org/10.1038/bjc.2011.388
104. Cheng Y, He C, Wang M et al (2019) Targeting epigenetic regulators for cancer therapy: mechanisms and advances in clinical trials. Signal Transduct Target Ther. https://doi.org/10.1038/s41392-019-0095-0
105. Chang JC (2016) Cancer stem cells. Medicine. https://doi.org/10.1097/2FMD.0000000000004766
106. Peitzsch C, Tyutyunnykova A, Pantel K, Dubrovska A (2017) Cancer stem cells: the root of tumor recurrence and metastases. Semin Cancer Biol 44:10–24. https://doi.org/10.1016/j.semcancer.2017.02.011
107. Su JM, Li X-N, Thompson P et al (2010) Phase 1 study of valproic acid in pediatric patients with refractory solid or CNS tumors: a children's oncology group report. Clin Cancer Res 17:589–597. https://doi.org/10.1158/1078-0432.CCR-10-0738
108. Su JMF, Murray JC, Mcnall-Knapp RY et al (2020) A phase 2 study of valproic acid and radiation, followed by maintenance valproic acid and bevacizumab in children with newly diagnosed diffuse intrinsic pontine glioma or high-grade glioma. Pediatr Blood Cancer. https://doi.org/10.1002/pbc.28283
109. Masoudi A, Elopre M, Amini E, Nagel ME, Ater JL, Gopalakrishnan V, Wolff JE (2008) Influence of valproic acid on outcome of high-grade gliomas in children. Anticancer Res 28:2437–2442
110. Tilburg CMV, Milde T, Witt R et al (2019) Phase I/II intra-patient dose escalation study of vorinostat in children with relapsed solid tumor, lymphoma, or leukemia. Clin Epigenetics. https://doi.org/10.1186/s13148-019-0775-1
111. Hummel TR, Wagner L, Ahern C et al (2013) A pediatric phase 1 trial of vorinostat and temozolomide in relapsed or refractory primary brain or spinal cord tumors: a children's oncology group phase 1 consortium study. Pediatr Blood Cancer 60:1452–1457. https://doi.org/10.1002/pbc.24541
112. Dubois SG, Groshen S, Park JR et al (2015) Phase I study of vorinostat as a radiation sensitizer with 131I-metaiodobenzylguanidine (131I-MIBG) for patients with relapsed or refractory neuroblastoma. Clin Cancer Res 21:2715–2721. https://doi.org/10.1158/1078-0432.CCR-14-3240
113. National Cancer Institute (NCI) (2016) A phase 1 study of entinostat, an oral histone deacetylase inhibitor, in pediatric patients with recurrent or refractory solid

tumors, including CNS tumors and lymphoma. Identifier NCT02780804. Retrieved from https://clinicaltrials.gov/ct2/show/NCT02780804
114. University Hospital Heidelberg (2019) Exploratory multinational phase I/II combination study of nivolumab and entinostat in children and adolescents with refractory high-risk malignancies. Identifier NCT03838042. Retrieved from https://clinicaltrials.gov/ct2/show/NCT03838042
115. National Cancer Institute (NCI) (2010) A randomized phase II/III study of vorinostat and local irradiation OR temozolomide and local irradiation OR bevacizumab and local irradiation followed by maintenance bevacizumab and temozolomide in children with newly diagnosed high-grade gliomas. Identifier NCT01236560. Retrieved from https://clinicaltrials.gov/ct2/show/NCT01236560
116. Dana-Farber, Cancer Institute (2020) Phase 1 trial of marizomib alone and in combination with panobinostat for children with diffuse intrinsic pontine glioma. Identifier NCT04341311. Retrieved from https://clinicaltrials.gov/ct2/show/NCT04341311
117. The Hospital for Sick Children (2017) Phase I/Ib trial of combined 5'azacitidine and carboplatin for recurrent/refractory pediatric brain and solid tumors. Identifier NCT03206021. Retrieved from https://clinicaltrials.gov/ct2/show/NCT03206021
118. Ganesan A, Arimondo PB, Rots MG et al (2019) The timeline of epigenetic drug discovery: from reality to dreams. Clin Epigenetics. https://doi.org/10.1186/s13148-019-0776-0
119. Italiano A et al (2018) Tazemetostat, an EZH2 inhibitor, in relapsed or refractory B-cell non-Hodgkin lymphoma and advanced solid tumours: a first-in-human, open-label, phase 1 study. Lancet Oncol 19:649–659. https://doi.org/10.1016/S1470-2045(18)30145-1
120. Janssen Research & Development, LLC (2018) A Phase 1, first-in-human, open-label study of the safety, pharmacokinetics, and pharmacodynamics of JNJ-64619178, an inhibitor of protein arginine methyltransferase 5 (PRMT5) in subjects with advanced cancers. Identifier NCT03573310. Retrieved from https://clinicaltrials.gov/ct2/show/NCT03573310
121. Dartmouth-Hitchcock Medical Center (2013) Evaluating the expression levels of microRNA-10b in patients with gliomas. Identifier NCT01849952. Retrieved from https://clinicaltrials.gov/ct2/show/NCT01849952
122. Fouladi M et al (2010) Pediatric phase I trial and pharmacokinetic study of vorinostat: a children's oncology group phase I consortium report. J Clin Oncol 28:3623–3629. https://doi.org/10.1200/JCO.2009.25.9119
123. Muscal JA et al (2013) A phase I trial of vorinostat and bortezomib in children with refractory or recurrent solid tumors: a children's oncology group phase I consortium study (ADVL0916). Pediatr Blood Cancer 60:390–395. https://doi.org/10.1002/pbc.24271
124. M.D. Anderson Cancer Center (2015) Vorinostat and temsirolimus with or without radiation therapy in treating younger patients with newly diagnosed or progressive diffuse intrinsic pontine glioma. Identifier NCT02420613. Retrieved from https://clinicaltrials.gov/ct2/show/NCT02420613
125. New Approaches to Neuroblastoma Therapy Consortium (2017) MIBG with dinutuximab +/− vorinostat. Identifier NCT03332667. Retrieved from https://clinicaltrials.gov/ct2/show/NCT03332667
126. Jubilant DraxImage Inc. (2018) A study of therapeutic iobenguane (131-I) and vorinostat for recurrent or progressive high-risk neuroblastoma subjects (OPTIMUM). Identifier NCT03561259. Retrieved from https://clinicaltrials.gov/ct2/show/NCT03561259
127. Pinto N et al (2018) Phase I study of vorinostat in combination with isotretinoin in patients with refractory/recurrent neuroblastoma: a new approaches to neuroblastoma therapy (NANT) trial. Pediatr Blood Cancer 65:e27023. https://doi.org/10.1002/pbc.27023
128. New York Medical College (2020) Vorinostat in combination with chemotherapy in relapsed/refractory solid tumors and CNS malignancies (NYMC195). Identifier NCT04308330. Retrieved from https://clinicaltrials.gov/ct2/show/NCT04308330
129. Leary SES et al (2022) Vorinostat and isotretinoin with chemotherapy in young children with embryonal brain tumors: a report from the pediatric brain tumor consortium (PBTC-026). Neuro Oncol 24:1178–1190. https://doi.org/10.1093/neuonc/noab293
130. Witt O et al (2012) Phase I/II intra-patient dose escalation study of vorinostat in children with relapsed solid tumor, lymphoma or leukemia. Klin Padiatr 224:398–403. https://doi.org/10.1186/s13148-019-0775-1
131. Peters KB et al (2018) Phase I/II trial of vorinostat, bevacizumab, and daily temozolomide for recurrent malignant gliomas. J Neurooncol 137:349–356. https://doi.org/10.1007/s11060-017-2724-1
132. Su JM et al (2022) Phase I/II trial of vorinostat and radiation and maintenance vorinostat in children with diffuse intrinsic pontine glioma: a children's oncology group report. Neuro Oncol 24:655–664. https://doi.org/10.1093/neuonc/noab188
133. Wake Forest University Health Sciences (2015) Pediatric precision laboratory advanced neuroblastoma therapy (PEDS-PLAN). Identifier NCT02559778. Retrieved from https://clinicaltrials.gov/ct2/show/NCT02559778
134. DuBois SG et al (2021) Randomized phase II trial of MIBG versus MIBG, vincristine, and irinotecan versus MIBG and vorinostat for patients with relapsed or refractory neuroblastoma: a report from NANT consortium. J Clin Oncol 39:3506–3514. https://doi.org/10.1200/JCO.21.00703
135. Wolff JE et al (2008) Valproic acid was well tolerated in heavily pretreated pediatric patients with high-grade glioma. J Neurooncol 90:309–314. https://doi.org/10.1007/s11060-008-9662-x
136. Felix FH, Trompieri NM, de Araujo OL, da Trindade KM, Fontenele JB (2011) Potential role for valproate in the treatment of high–risk brain tumors of childhood-results from a retrospective observational cohort study. Pediatr Hematol Oncol 28:556–570. https://doi.org/10.3109/08880018.2011.563774
137. Wolff JE et al (2012) Treatment of recurrent diffuse intrinsic pontine glioma: the MD anderson cancer center experience. J Neurooncol 106:391–397. https://doi.org/10.1007/s11060-011-0677-3
138. Su JM et al (2020) A phase 2 study of valproic acid and radiation, followed by maintenance valproic acid and bevacizumab in children with newly diagnosed diffuse intrinsic pontine glioma or high-grade glioma. Pediatr Blood Cancer 67:e28283. https://doi.org/10.1002/pbc.28283
139. University of California, San Francisco (2021) Combination therapy for the treatment of diffuse midline gliomas. Identifier NCT05009992. Retrieved from https://clinicaltrials.gov/ct2/show/NCT05009992
140. University of Göttingen (2017) International Cooperative Phase III Trial of the HIT-HGG Study Group (HIT-HGG-2013) (HIT-HGG-2013). Identifier NCT03243461. Retrieved from https://clinicaltrials.gov/ct2/show/NCT03243461
141. Bukowinski A et al (2021) A phase 1 study of entinostat in children and adolescents with recurrent or refractory solid tumors, including CNS tumors: trial ADVL1513, pediatric early phase-clinical trial network (PEP-CTN). Pediatr Blood Cancer 68:e28892. https://doi.org/10.1002/pbc.28892
142. van Tilburg CM et al (2020) INFORM2 NivEnt: the first trial of the INFORM2 biomarker driven phase I/II trial series: the combination of nivolumab and entinostat in children and adolescents with refractory high-risk malignancies. BMC Cancer 20:523. https://doi.org/10.1186/s12885-020-07008-8

143. Pediatric Brain Tumor Consortium (2016) Trial of panobinostat in children with diffuse intrinsic pontine glioma (PBTC-047). Identifier NCT02717455. Retrieved from https://clinicaltrials.gov/ct2/show/NCT02717455
144. The University of Texas Health Science Center, Houston (2020) infusion of panobinostat (MTX110) into the fourth ventricle in children and adults with recurrent medulloblastoma. Identifier NCT04315064. Retrieved from https://clinicaltrials.gov/ct2/show/NCT04315064
145. Cheng-Chia (Fred) Wu (2021) Non-invasive focused ultrasound (FUS) with oral panobinostat in children with progressive diffuse midline glioma (DMG). Identifier NCT04804709. Retrieved from https://clinicaltrials.gov/ct2/show/NCT04804709
146. Luca Szalontay (2020) CED of MTX110 newly diagnosed diffuse midline gliomas. Identifier NCT04264143. Retrieved from https://clinicaltrials.gov/ct2/show/NCT04264143
147. National Cancer Institute (NCI) (2017) Targeted therapy directed by genetic testing in treating pediatric patients with relapsed or refractory advanced solid tumors, non-hodgkin lymphomas, or histiocytic disorders (The pediatric MATCH screening trial). Identifier NCT03155620. Retrieved from https://clinicaltrials.gov/ct2/show/NCT03155620
148. National Cancer Institute (NCI) (2017) Tazemetostat in treating patients with relapsed or refractory advanced solid tumors, non-hodgkin lymphoma, or histiocytic disorders With EZH2, SMARCB1, or SMARCA4 gene mutations (a pediatric MATCH treatment trial). Identifier NCT03213665. Retrieved from https://clinicaltrials.gov/ct2/show/NCT03213665
149. George RE et al (2010) Phase I study of decitabine with doxorubicin and cyclophosphamide in children with neuroblastoma and other solid tumors: a children's oncology group study. Pediatr Blood Cancer 55:629–638. https://doi.org/10.1002/pbc.22607
150. Krishnadas DK et al (2015) A phase I trial combining decitabine/dendritic cell vaccine targeting MAGE-A1, MAGE-A3 and NY-ESO-1 for children with relapsed or therapy-refractory neuroblastoma and sarcoma. Cancer Immunol Immunother 64:1251–1260. https://doi.org/10.1007/s00262-015-1731-3
151. AbbVie (2017) A Study of the Safety and Pharmacokinetics of Venetoclax in Pediatric and Young Adult Patients With Relapsed or Refractory Malignancies. Identifier NCT03236857. Retrieved from https://clinicaltrials.gov/ct2/show/NCT03236857
152. Fenaux P et al (2009) Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: a randomised, open-label, phase III study. Lancet Oncol 10:223–232. https://doi.org/10.1016/S1470-2045(09)70003-8
153. Kantarjian H et al (2006) Decitabine improves patient outcomes in myelodysplastic syndromes: results of a phase III randomized study. Cancer 106:1794–1803. https://doi.org/10.1002/cncr.21792
154. Mann BS, Johnson JR, Cohen MH, Justice R, Pazdur R (2007) FDA approval summary: vorinostat for treatment of advanced primary cutaneous T-cell lymphoma. Oncologist 12:1247–1252. https://doi.org/10.1634/theoncologist.12-10-1247
155. Piekarz RL et al (2009) Phase II multi-institutional trial of the histone deacetylase inhibitor romidepsin as monotherapy for patients with cutaneous T-cell lymphoma. J Clin Oncol 27:5410–5417. https://doi.org/10.1200/JCO.2008.21.6150
156. O'Connor OA et al (2015) Belinostat in patients with relapsed or refractory peripheral T-Cell lymphoma: results of the pivotal phase II BELIEF (CLN-19) study. J Clin Oncol 33:2492–2499. https://doi.org/10.1200/JCO.2014.59.2782
157. Laubach JP et al (2021) Efficacy and safety of oral panobinostat plus subcutaneous bortezomib and oral dexamethasone in patients with relapsed or relapsed and refractory multiple myeloma (PANORAMA 3): an open-label, randomised, phase 2 study. Lancet Oncol 22:142–154. https://doi.org/10.1016/S1470-2045(20)30680-X
158. Santos FP, Kantarjian H, Garcia-Manero G, Issa JP, Ravandi F (2010) Decitabine in the treatment of myelodysplastic syndromes. Expert Rev Anticancer Ther 10:9–22. https://doi.org/10.1586/era.09.164
159. Götze K et al (2010) Azacitidine for treatment of patients with myelodysplastic syndromes (MDS): practical recommendations of the German MDS study group. Ann Hematol 89:841–850. https://doi.org/10.1007/s00277-010-1015-0
160. Duvic M et al (2009) Evaluation of the long-term tolerability and clinical benefit of vorinostat in patients with advanced cutaneous T-cell lymphoma. Clin Lymphoma Myeloma 9:412–416. https://doi.org/10.3816/CLM.2009.n.082

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CAPÍTULO II

Valproic acid modulation of stemness is dependent of medulloblastoma molecular subgroups

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Valproic acid modulation of stemness is dependent of medulloblastoma molecular subgroups

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Abstract

Medulloblastoma (MB) is the most common malignant childhood brain tumor. Recurrent and metastatic disease, which occurs in approximately 30% of patients, is the main cause of death and may be related to the presence of cancer stem cells (CSCs). Epigenetic mechanisms are major drivers for the establishment and maintenance of CSC, and inhibition of histone deacetylase enzymes (HDAC) has shown potential as a therapeutic strategy to target this cell population. Here, we found that HDAC inhibitor Valproic acid (VPA) enhances histone acetylation and decreases cell and CSC viability. VPA main effects are related to the increase in neuronal differentiation and decrease in stemness pathways. Specific molecular mechanisms of VPA seem to be dependent on MB molecular subgroups. In Sonic Hedgehog (SHH) MB, VPA modulates the cell cycle through the MYC-P21-SOX2 axis, whereas in Group 3 VPA regulates the Notch1 pathway. These findings suggest that HDAC inhibiting by VPA could be a novel effective approach against cell with stemness features and high malignant traits.

Key words: Medulloblastoma, Cancer stem cells, Valproic acid, Stemness

Introduction

Medulloblastoma (MB) is the most common malignant childhood brain tumor [1]. MB arises from neural stem cells (NSCs) or cerebellar granule neuron precursors (GNPs) that undergo genetic and epigenetic alterations [2-3]. Genomic, epigenomic, and transcriptional analyses have shown that MB is a heterogeneous tumor that differs in molecular, clinical, and prognosis features [4]. The World Health Organization (WHO) 2016 classification established that MB is comprised of distinct molecular subgroups: Wingless (WNT) MB, Sonic Hedgehog (SHH) MB/TP53 wild type, SHH MB/ TP53 mutated, group 3, and group 4 [5].

Even though there were advancements in MB therapies in the last decades, metastatic and recurrent tumors are still a challenge. Relapse MB tumors occur in approximately 30% of patients and have high mortality rates [6]. According to the cancer stem cell (CSC) theory, tumor heterogeneity can be explained by the presence of a specific subpopulation of cells, known as cancer stem cells [7]. CSC have stem cell features such as self-renewal and differentiation potential. Studies show that CSCs are more resistant to treatment and have higher metastatic capabilities [8-10]. MB tumor-derived cells and neurospheres express CD133, SOX2, and BMI1, markers related to neural progenitors and stem cell features [11]. Moreover, CD133 positive (CD133+) MB cells were able to initiate tumors that present the same characteristic that the patient's original tumors when implanted in NOD-SCID mice [12], supporting the premise that CSC contributes to MB initiation and recurrence.

Epigenetic mechanisms are major drivers for the establishment and maintenance of CSC. Most common mutations found in cancer can be related to epigenetic regulators [13]. Thus, an altered epigenetic profile enables cellular reprogramming that contributes to an aberrant activating of stem cell pathways promoting the acquisition of uncontrolled self-renewal [14-15]. Moreover, the choice between an undifferentiated and differentiated state can be controlled by alterations in the epigenome [16]. Valproic acid (VPA) is an anticonvulsant drug that also has an effect as an epigenetic modulator capable of inhibiting histone deacetylases (HDAC). VPA inhibits specifically HDAC class I and IIa (HDAC 1–5, 7) and consequently enhances the histone acetylation landscape [17]. In MB, VPA was associated with changes in cell cycle progression, senescence, and apoptosis [18]. In Glioblastoma stem cells, VPA downregulates the expression of stemness genes CD133, Nanog, OCT4, and enhances differentiation markers [19]. Hence, suggesting that VPA

modulates the epigenome and contributes to CSC fate. Here, we elucidate the role of VPA in the stemness maintenance of MB cancer stem cells.

Materials and Methods

Cell lines and cell culture conditions

MB cell lines D283 (ATCC® HTB-185™) and Daoy (ATCC® HTB-186™) were originally obtained from the American Type Culture Collection (ATCC, Rockville, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM low glucose, Gibco®) containing 10% (v/v) fetal bovine serum (FBS, Gibco®), 1% (v/v) penicillin-streptomycin solution (10,000U/mL, Gibco®) and 0.1% (v/v) amphotericin B (250 μg/mL; Gibco®). Cells were cultured at 37°C in a humidified incubator under 5% CO_2. Experiments were conducted in exponentially growing cell cultures.

Drug Treatment

Histone deacetylase inhibitor Valproic acid (VPA; Santa Cruz Biotechnology - CAS 1069-66-5) was dissolved in sterile water to a stock concentration of 0.3 M.

Cell viability

Cells were treated with VPA (0.5, 1.0, 2.5, 5.0, 10.0 or 20.0 mM) for 48 and 72 hours. MB cells were seeded at 3000 cells/well in 96 wells plates and, after VPA exposure, cells were detached with trypsin-EDTA (Gibco®) and counted in a Neubauer chamber with trypan for viability measurement. The doses of VPA were chosen based on previous *in vitro* studies using cultured medulloblastoma and glioblastoma cell lines [18, 20]. Experiments were conducted in three biological replicates. For IC_{50} determination, cell viability data were fitted in a dose-response curve (Graphpad Prism v. 6.0).

Sphere Formation Assay

Sphere formation assay was used as a model to study cancer stem cells and experiment parameters were on the bases of previous *in vitro* studies [21-22]. To analyze the effects of VPA during sphere formation, MB cells were dissociated with trypsin-EDTA into cell suspension and seeded at 500 cells/well in 24-well plates. Agarose solution (1%) was

used to overcome cell adherence. Cells were cultured in serum-free sphere-induction medium, containing DMEM/F12 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 during 5 days as described [22]. Cells were monitored daily until sphere formation. To analyze effects during sphere formation, VPA (1.0, 2.5, 5.0, 10.0 or 20.0 mM) was added at the first day of sphere induction and sphere size was measured after a period of 5 days. To verify VPA modulation after sphere formation, MB cells were dissociated with trypsin-EDTA into cell suspension and seeded at 500 or 1000 cells/well in 24-well ultra-low attachment plates (Corning®) in serum-free sphere-induction medium. After 5 days, VPA was added at a final concentration equal to IC_{50} dose (D283, 2.3 mM; Daoy, 2.2 mM). Spheres size and number were analyzed after period of 48 hours of VPA exposure. Images were taken an inverted microscope at ×5 magnification. Sphere size was measured using ImageJ (National Institutes of Health, Bethesda, USA). A sphere was considered if it had at least 15 cells. Experiments were conducted in three biological replicates.

Reverse Transcriptase Polymerase Chain Reaction (RT-qPCR)

The mRNA expression of target genes was analyzed by RT-qPCR. RNA was extracted from MB spheres and monolayer cells using ReliaPrep™ RNA Miniprep System (Promega), in accordance with the manufacturer's instructions and quantified in NanoDrop (Thermo Fisher Scientific). The cDNA was obtained using the GoScript Reverse System (Promega) also according to the manufacturer's instructions. The mRNA expression levels of *CDKN1A, ENO2, HES1, Nestin*, *NOTCH1, RBFOX3, SOX2* and *TUBB3* were quantified using PowerUp SYBR Green Master Mix (Applied Biosystems, Thermo Fisher Scientific). The primers used for RT-qPCR amplification were designed according to literature and are shown in Table 1. The expression of *ACTB* was measured as control.

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

Gene	Primer Forward (5'-3')	Primer Reverse (5'-3')
ACTB	AAACTGGAACGGTGAAGGTG	AGAGAAGTGGGGTGGCTTTT
CDKN1A	ACTCTCAGGGTCGAAAACGG	CTTCCTGTGGGCGGATTAGG

ENO2	AGCCTCTACGGGCATCTATGA	TTCTCAGTCCCATCCAACTCC
HES1	AGTGAAGCACCTCCGGAAC	TCACCTCGTTCATGCACTC
Nestin	GATCGCTCAGGTCCTGGAAG	GGGGTCCTAGGGAATTGCAG
NOTCH1	AAGCTGCATCCAGAGGCAAAC	TGGCATACACACTCCGAGAACAC
RBFOX3	CCAGGCTCCGAGGCCAGCACAC	TGTAGGGTCGGAGGGGTGGAG
SOX2	CAGCTCGCAGACCTACATGA	GGGAGGAAGAGGTAACCACAG
TUBB3	CTCAGGGGCCTTTGGACATC	CAGGCAGTCGCAGTTTTCAC

Cell Cycle

To assess cell cycle, MB treated with IC50 doses of VPA. After 48 hours of exposure cells were detached, centrifuged and washed with PBS twice. The cells were then resuspended in 50 µg/ml propidium iodide (Sigma-Aldrich, St. Louis, Mo., USA) in 0.1% Triton X-100 in 0.1% sodium citrate solution and incubated on ice for 15 min. The cells were analyzed by flow cytometry (Attune® Applied Biosystems) and 20,000 events were collected per sample.

Western Blot

Monolayer MB cells VPA-treated, and control were lysed with 1X Lysis Buffer (Cell Lysis Buffer, Cell Signaling Technology), and protein was quantified using the Bradford protein assay (Pierce, Thermo Scientific, Waltham, USA). For blotting, 40 µg of protein were separated by SDS-PAGE and transferred to a PVDF membrane. After 1 h with blocking solution (5% milk in TTBS), the membrane was incubated overnight at 4 °C with primary antibodies against p21 (1:200; Santa Cruz Biotechnology) and β-actin (1:2000; Santa Cruz Biotechnology) as loading control. Incubation of primary antibodies was followed by incubation with the secondary antibody adequate to each primary antibody for 1 h. Chemiluminescence was detected using ECL Western Blotting substrate (Pierce, Thermo Scientific) and analyzed using iBright (Thermo Fisher Scientific). Immunodetection signals were analyzed using ImageJ (National Institutes of Health, Bethesda, USA).

Immunofluorescence

The immunofluorescence assays were performed on control and VPA-treated MB monolayer and spheres using primary antibody against histone H3K9ac (1:3000; Abcam)

histone H3 (1:250; Thermo Fischer Scientific), SOX2 (1:1000; Abcam). Alexa Fluor 488-conjugated goat anti-rabbit (1:1000; Abcam) and Alexa Fluor 594-conjugated anti-mouse (1:1000; Abcam) was used as the secondary antibody. Flourished with DAPI (Sigma Aldrich) was used to counterstain the nuclei.

Briefly, cells were seeded into coverslips treated with Poli-L-Lysine solution 0.01% (Sigma Aldrich) and treated with VPA for 48 hours, while spheres were moved to coverslips treated with Poli-L-Lysine solution 0.01% after treatment. Monolayer cells and spheres were washed with Phosphate-Buffered Saline (PBS), fixed with methanol for 5 min at room temperature (RT) and washed 2x with ice cold PBS. Coverslips were incubated for 30 min at RT in blocking solution (1% of Bovine Serum Albumin (BSA), 0.1% Tween 20 in PBS) and with the primary antibodies at 4°C overnight. Then, the coverslips were rinsed three times with PBS and incubated with secondary fluorescent antibodies for 1 h at RT. Cells and spheres were then washed with PBS and coverslips were mounted. Fluorescent spheres were examined using Leica microscope 5 or 10 x amplification.

Statistics

Data are shown as mean ± standard error of mean (SEM). Statistical analyses were performed by either Student's T-test, when comparing two groups, or one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc tests for multiple comparisons. Experiments were replicated at least three times; P values under 0.05 were considered significant. The GraphPad Prism 6 software (GraphPad Software, San Diego, USA) was used for analyses.

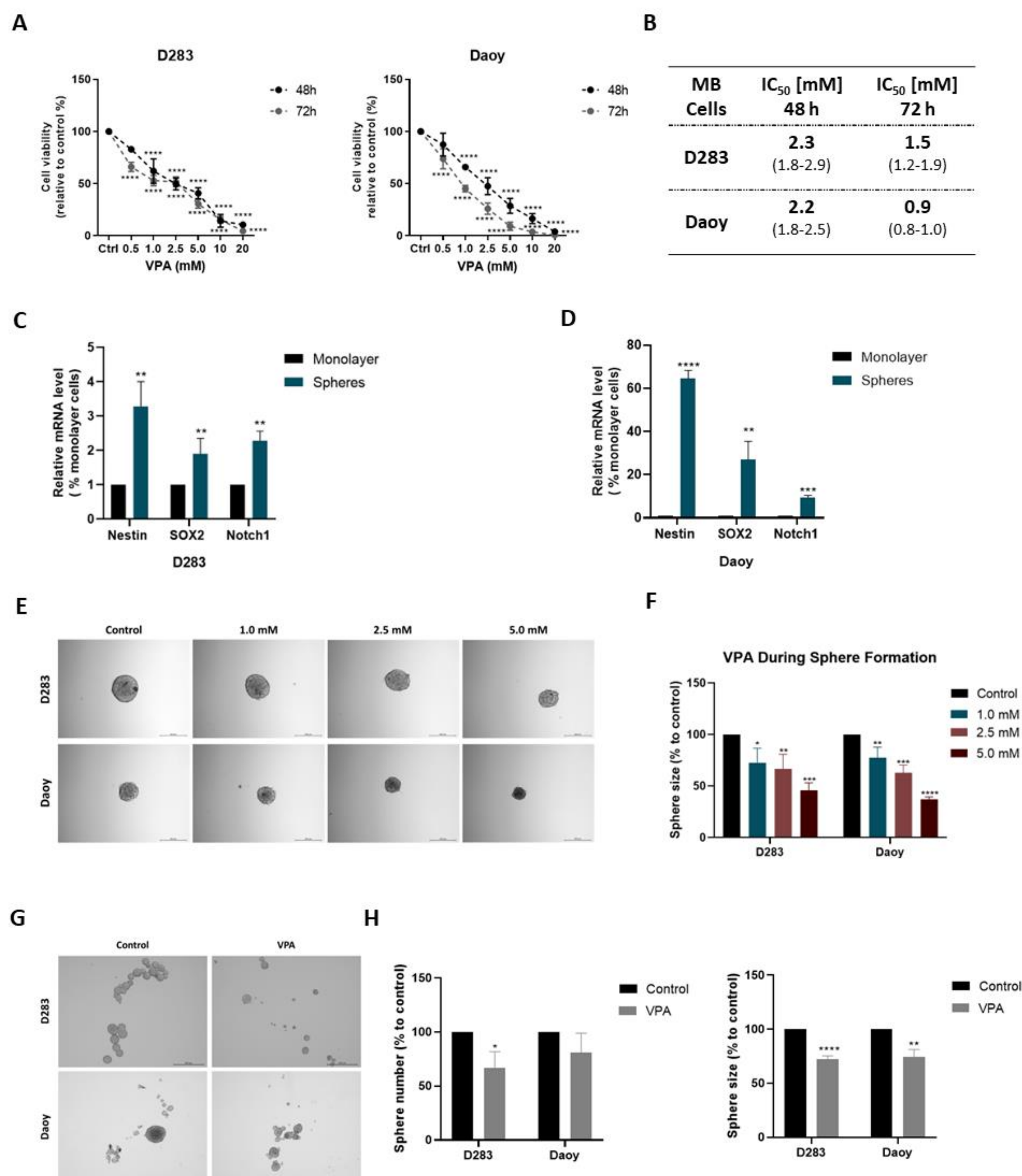
Results

VPA decreases MB cell and neurospheres viability

To evaluate the effects of VPA inhibition on MB cell viability, we exposed the cells to different concentrations of VPA (0.5; 1.0; 2.5; 5.0; 10.0; 20.0 mM) for 48 or 72 hours. VPA was able to reduce MB cells viability in a dose-dependent manner (Fig. 1A). Fifty percent inhibition of growth (IC_{50}) was determined and both MB cell lines presented similar responses to VPA inhibition (2.3 mM for D283 and 2.2 mM for Daoy cells) (Fig. 1B). Since exposure to VPA had only a slight increase in VPA inhibition capability, following experiments were conducted using 48 hours of exposure time.

Neurosphere assays are widely used to investigate the proliferation of MB cancer stem cells [21, 23]. First, we verified the expression of stemness genes Nestin, SOX2, and NOTCH1 in D283 and Daoy neurospheres compared to monolayer cells. After 7 days of culturing cells in appropriated medium for expansion of tumor stem cells, D283 and Daoy neurospheres had an increase of transcriptional levels of all evaluated stemness genes (2.2-fold, $p < 0.01$ in Nestin; 0.9-fold, $p < 0.01$ in SOX2; 1.2-fold, $p < 0.01$ in NOTCH1) (63.4-fold, $p < 0.0001$ in Nestin; 26-fold, $p < 0.01$ in SOX2; 8.5-fold, $p < 0.001$ in NOTCH1), respectively (Fig. 1C-D). These data suggest that the neurosphere assay is able to enrich the CSC population in MB cells.

To elucidate whether VPA could impair MB neurosphere formation, we measured neurosphere size after growth in the presence of VPA (1.0; 2.5; 5.0 mM). VPA was able to impair neurosphere formation in both MB cell lines. VPA at all concentrations tested significantly reduced the sphere size after 5 days of VPA exposure compared to controls (Fig. 1E-F). We also examined if VPA could be capable of reducing sphere size and the number of MB neurospheres. After 5 days of CSC induction, MB neurospheres were treated with VPA at the estimated IC_{50}s (2.3 mM for D283 and 2.2 mM for Daoy) for 48 hours. VPA was able to reduce D283 neurosphere number and size (33%, $P < 0.05$; 27%, $P < 0.0001$; respectively). In Daoy neurospheres, we found that VPA was able to reduce neurosphere size (25.5%, $P < 0.01$) (Fig 1G-H).



MB Cells	IC_{50} [mM] 48 h	IC_{50} [mM] 72 h
D283	2.3 (1.8-2.9)	1.5 (1.2-1.9)
Daoy	2.2 (1.8-2.5)	0.9 (0.8-1.0)

Figure 1: VPA decreases MB cell and neurospheres viability. **A**. MB cells were treated with a range of VPA concentrations (0.5; 1.0; 2.5; 5.0; 10.0; 20.0 mM) for 48 and 72 hours and cell viability was measured by trypan exclusion assay. **B**. IC_{50} concentrations of VPA for MB cells with 95% confidence interval (CI). **C-D**. Relative mRNA levels of Nestin, SOX2 and NOTCH1 in MB monolayer cells and neurospheres were verified using RT-qPCR. **E**. VPA effect on MB neurospheres formation after 5 days of VPA exposure. **F**. MB neurospheres size relative to control neurospheres. **G**. After 5 days of neurospheres formation VPA was added and evaluated after 48 hours. MB neurospheres number and size relative to control neurospheres. All images were taken in an inverted microscope with 5X amplification. Scale bar 500 µm. Results represent the mean ± SD of three independent

experiments; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 compared to controls or monolayer cells.

To confirm that VPA effects were due to an increase in histone acetylation, we verified acetylation on histone 3 lysine 9 residue (H3K9ac) by immunofluorescence assay. We found that VPA enhances the histone acetylation profile in MB cells and neurospheres (Fig. 2).

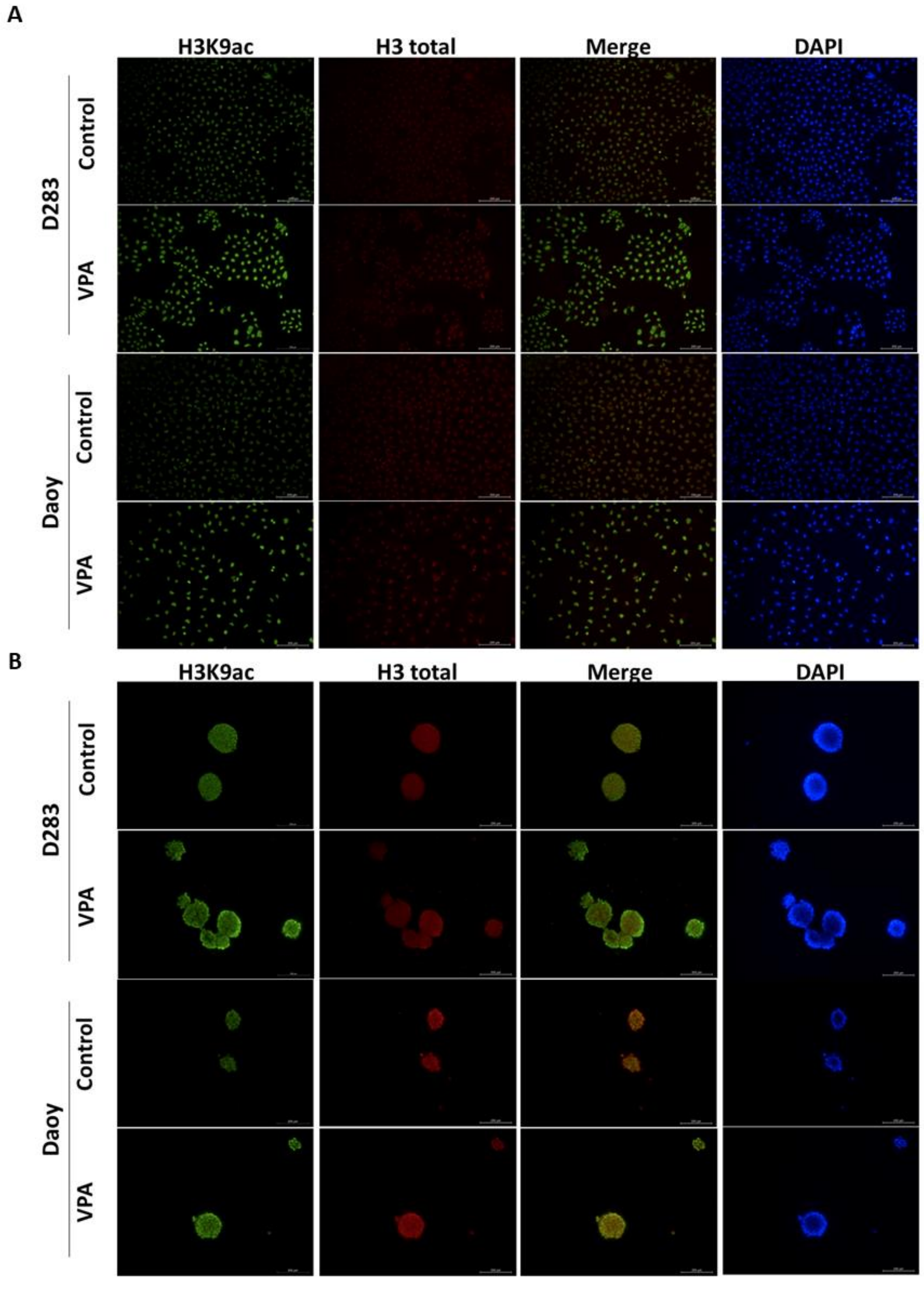


Figure 2: VPA enhances histone acetylation. Immunofluorescence assay using histone H3 lysine 9 acetylated residue (H3K9ac), total histone H3 total. MB cells and neurospheres were treated with VPA (D283 2.3mM; Daoy 2.2mM) for 48 hours. **A**. MB cells. **B.** MB neurospheres. Images were taken in an inverted microscope with 10X amplification. Scale bar 200 µm.

VPA increases p21 levels in SHH MB cells and neurospheres

To evaluate the effect of VPA on the arrest of the cell cycle, we first measured the expression of Cyclin-dependent kinase inhibitor 1 (CDKN1A), gene that encodes p21. VPA was able to increase the transcriptional levels of CDKN1A only in Daoy cells (3.2-fold, P <0.05 in Daoy cells). In addition, VPA exposure also caused an increase in p21 protein levels in Daoy cells (97 %, P <0.05) and a slight decrease in D283 cells (22%, P <0.05) (Fig 3B). VPA effects in p21 levels were accompanied by changes in cell cycle progression. Cell cycle analysis showed that VPA causes G1 arrest only in Daoy cells (P <0.05) (Fig. 3C). These results indicate that VPA modulation on the cell cycle pathway could be different depending on the MB molecular subgroup.

Next, we measured MYC transcriptional levels in D283 and Daoy cells and found that D283 has higher levels of MYC in comparison to Daoy (3.5-fold, p< 0.001) (Fig. 3D). VPA was able to decrease MYC levels only in Daoy cells (0.4-fold, p<0.05) (Fig. 3E). Studies indicate that MYC acts as a negative regulator of p21 [24-25]. Therefore, it is possible that VPA was not able to increase p21 levels in D283 cells due to high levels of MYC. In the MB CSC context, VPA reduced MYC in D283 neurospheres (0.3-fold, p<0.05) and Daoy neurospheres (0.8-fold, p<0.01) (Fig. 3F). Furthermore, VPA increases CDKN1A levels in both MB neurospheres (0.9-fold, P <0.05 in D283; 6-fold, P < 0.0001 in Daoy spheres) (Fig. 3F).

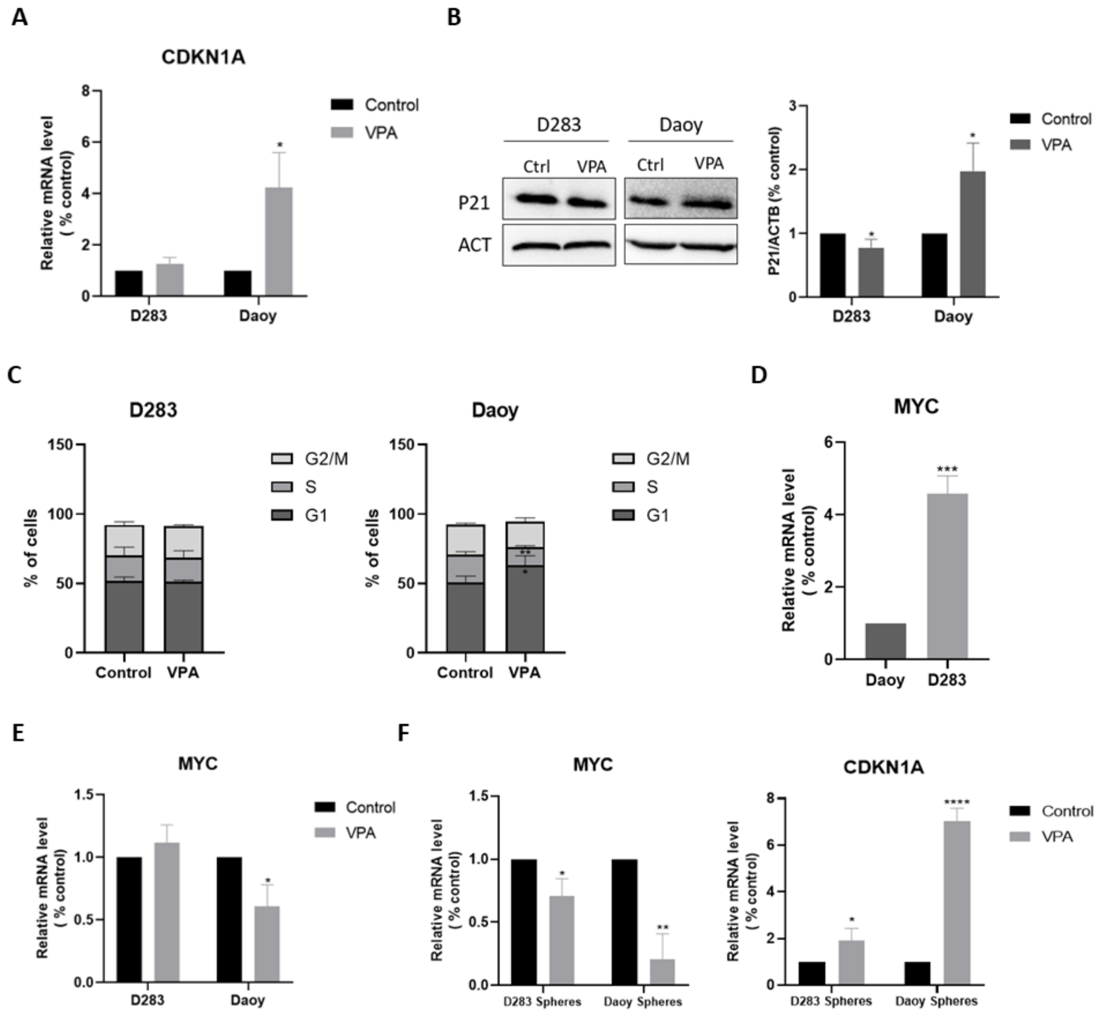


Figure 3: VPA increases p21 levels in SHH MB cells and neurospheres. A. Relative mRNA levels of CDKN1A in MB cells after VPA exposure was verified using RT-qPCR. **B** Western blot analysis of p21 in MB cells after VPA exposure**.** Relative Densitometric Unit (RDU) analysis normalized by ACTB and corrected by control. **C.** Cell cycle distribution of MB cells after VPA exposure. **D.** Relative mRNA levels of MYC between D283 and Daoy cells was verified using RT-qPCR. **E**. Relative mRNA levels of MYC and CDKN1A in MB cells and neurospheres after VPA exposure was verified using RT-qPCR. All experiments with D283 cells and neurospheres used 2,3 mM and Daoy cells and neurospheres used 2,2 mM of VPA, and a exposure time of 48 hour. Results represent the mean ± SD of three independent experiments; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 compared to controls.

VPA enhances neuronal differentiation markers in MB

To elucidate whether VPA could promote neuronal differentiation in MB cells and neurospheres, we measured the transcriptional levels of Tubulin Beta 3 Class III (TUBB3),

Enolase 2 (ENO2), and RNA Binding Fox-1 Homolog 3 (RBFOX3; the gene that encodes NeuN). RT-qPCR results indicated that VPA upregulates all three differentiation markers in D283 (4.6-fold, P < 0.01 in TUBB3; 1.5-fold, P < 0.01 in ENO2; 4.2-fold, P < 0.0001 in RBFOX3) and Daoy cells (0.4-fold, P < 0.01 in TUBB3; 1-fold, P < 0.01 in ENO2; 3.4-fold, P < 0.0001 in RBFOX3) (Fig. 4A-B). Moreover, VPA exposure promoted morphological changes similar to neuronal prolongation (Fig. 4C). In MB CSC, VPA increased all neuronal differentiation markers in D283 (1.9-fold, P < 0.0001 in TUBB3; 2-fold, P < 0.001 in ENO2; 14-fold, P < 0.0001 in RBFOX3 for D283 neurospheres) (Fig. 4D). In Daoy neurospheres, VPA upregulated TUBB3 (6.1-fold, P < 0.01) (Fig. 4E).

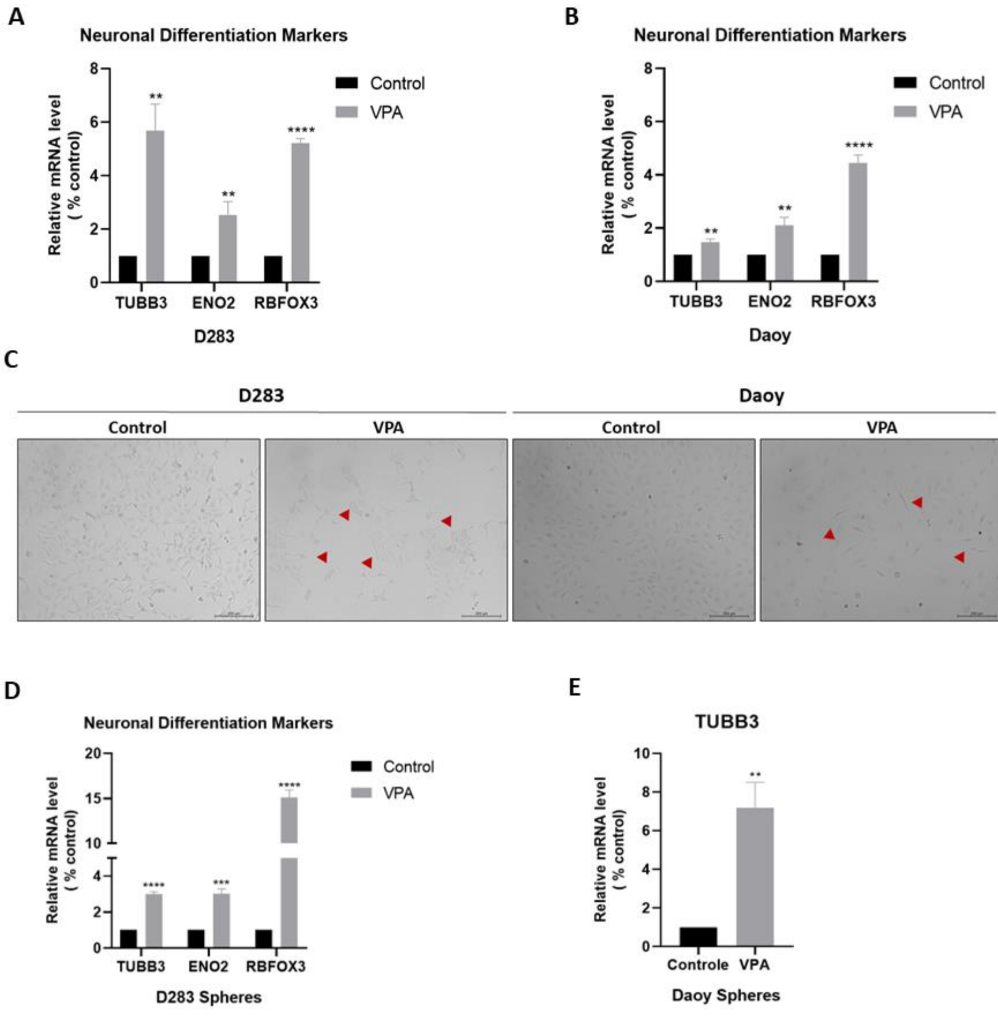


Figure 4: VPA enhances neuronal differentiation in MB. A-B and D-E. Relative mRNA levels of TUBB3, ENO2 and RBFOX3 in MB cells and neurospheres after VPA exposure

were verified using RT-qPCR. **C**. Representative images of MB cells exposed to VPA for 48 hours. Images were taken in an inverted microscope with 10X amplification. Scale bar 200 µm. Results represent the mean ± SD of three independent experiments; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 compared to controls.

VPA modulation of SOX2 is specific to SHH MB

Since VPA modulation can be correlated to a more differentiated state, we aimed to investigate whether VPA could cause changes in SOX2 levels. VPA exposure was able to reduce SOX2 transcriptional levels specifically in SHH MB cells, Daoy (0.4-fold, $p < 0.05$) (Fig. 5A). Daoy neurospheres also had a significant reduction of SOX2 levels (0.82-fold, $P < 0.001$) after VPA exposure (Fig. 5B). Moreover, protein levels of SOX2 were diminished in Daoy neurospheres (Fig. 5C). Neuronal progenitor marker, Nestin, is a target gene of SOX2. Hence, we sought to investigate if VPA exposure also resulted in Nestin modulation. VPA was capable of reducing Nestin transcriptional levels in Daoy cells (0.4-fold, $P < 0.05$) and neurospheres (0.87-fold, $P < 0.001$).

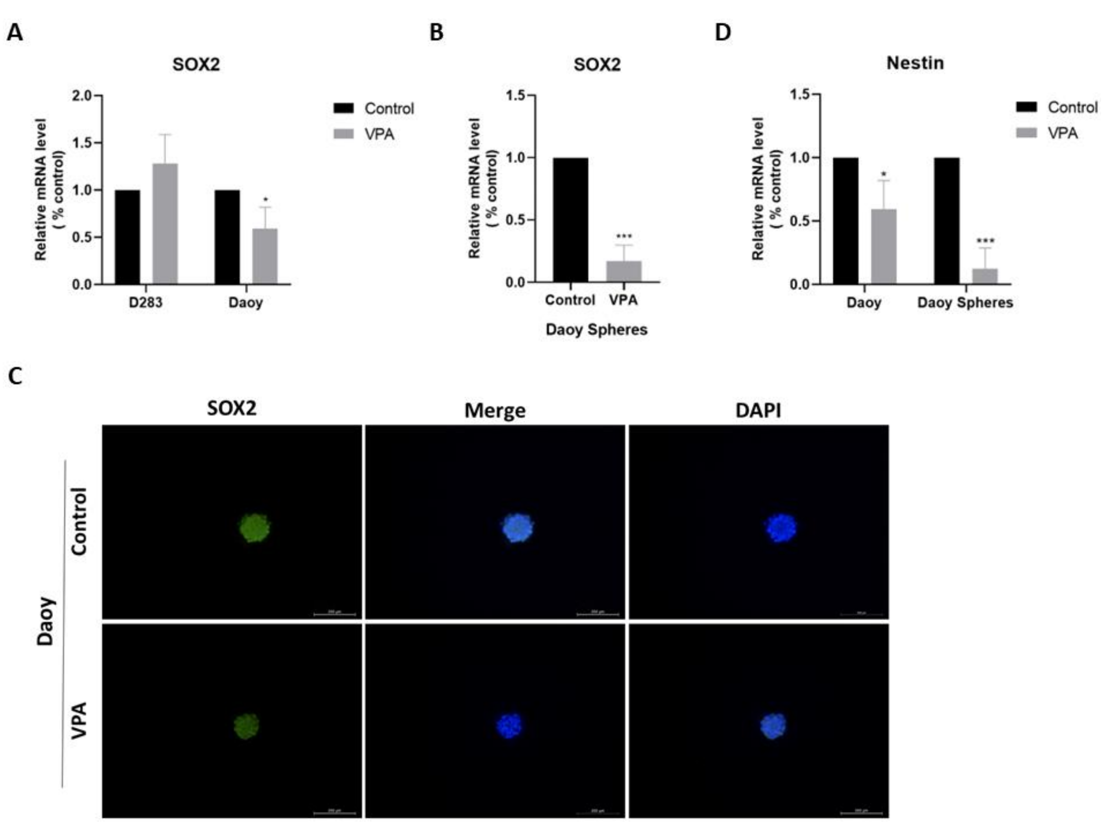


Figure 5: VPA modulation of SOX2 is specific to SHH MB. Relative mRNA levels of SOX2 after VPA exposure was verified using RT-qPCR. **A**. MB cells. **B**. Daoy neurospheres. **C.** Immunofluorescence assay using SOX2. Daoy neurospheres were treated with VPA (2.2mM) for 48 hours. Images were taken in an inverted microscope with 10X amplification. Scale bar 200 μm.**D**. Relative mRNA levels of Nestin in MB cells and neurospheres after VPA exposure was verified using RT-qPCR. Results represent the mean ± SD of three independent experiments; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 compared to controls.

VPA downregulates Notch1 in Group 3 MB cells and neurospheres

In Group 3 MB, Notch1 signaling pathway can be associated with self-renewal [26]. We sought to elucidate the VPA effect on Notch1 signaling pathway. RT-qPCR results indicated that VPA downregulates Notch1 levels only in D283 cells (0.18-fold, $p < 0.01$) (Fig. 6A). Notch1 downregulation was accompanied by modulation of Notch1 target gene, HES1. VPA exposure decreases HES1 transcriptional levels in D283 cells (0.26-fold, $p < 0.05$). In D283 neurospheres, VPA was able to reduce Notch1 (0.63-fold, $p < 0.01$) and HES1 (0.21-fold, $p < 0.05$) (Fig. 6B).

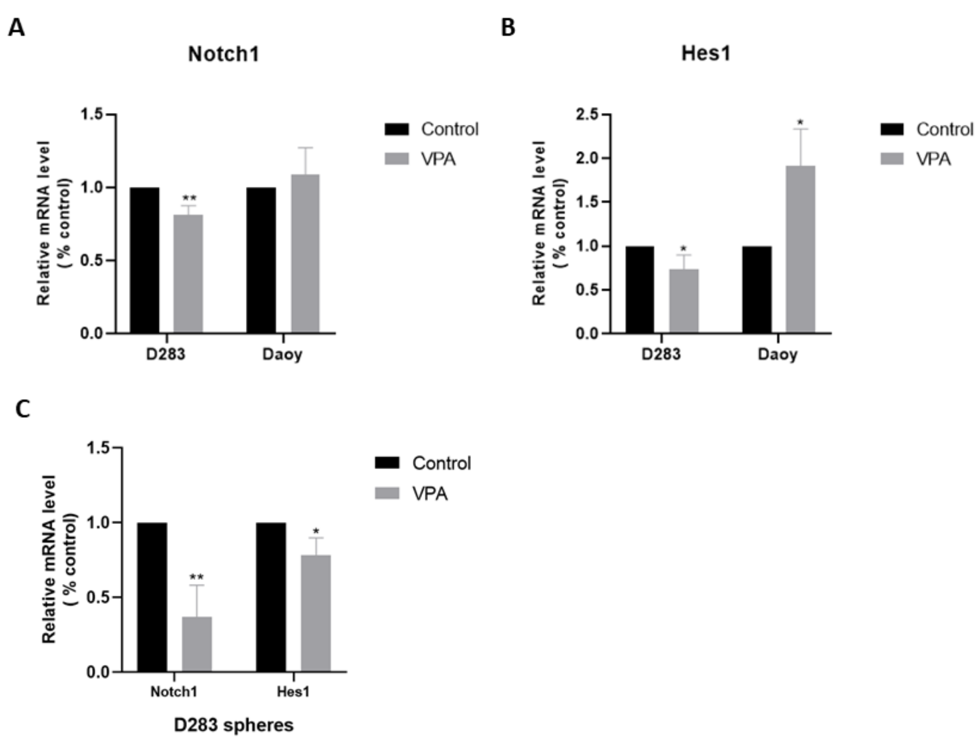


Figure 6: VPA downregulates Notch1 in Group 3 MB cells and neurospheres. Relative mRNA levels of Notch1 and Hes1after VPA exposure was verified using RT-qPCR. **A**. Notch1 levels in MB cells. **B**. HES1 levels in MB cells. **C**. Notch1 and HES1 levels in D283 neurospheres. Results represent the mean ± SD of three independent experiments; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$ compared to controls.

Discussion

MB is a pediatric tumor that presents high frequencies of mutation in epigenetic factors [13]. Dysregulation of the epigenome is also associated with MB cancer stem cells. Modulation of the histone acetylation landscape is considered a therapeutic alternative to alter stemness pathways responsible for the maintenance of CSC [27]. VPA is a well-known anti-convulsant drug that has an effect as an HDAC inhibitor [17]. Previous *in vitro* and *in vivo* studies indicate that VPA alters pathways related to cell cycle progression, senescence, apoptosis, and prolongs survival rates [18, 28]. Here, we found that VPA has an antiproliferative effect not only in MB cells but also in MB CSC (Fig. 1).

Reduction of MB cell and CSC viability can be associated with the increase of histone acetylation (Fig. 2). Hyperacetylation is related to the opening of the chromatin structure. VPA downregulates proteins essential to the chromatin state, such as SMCs (Structural Maintenance of chromatin), HP1 (Heterochromatin Protein-1), and DNMT1 (DNA methyl transferase-1) [29]. Therefore, VPA increase in histone acetylation contributes to an open chromatin state that enables several alterations in the epigenetic landscape. Here, we found that VPA changes the expression of genes that regulates cell cycle progression, differentiation, and stemness maintenance.

Cell cycle progression is an essential step to maintain cell viability and growth. The cyclin-dependent kinase inhibitor p21 (also known as p21WAF1/Cip1) acts as a repressor and contributes to cell cycle arrest [30]. VPA was capable of increasing CDKN1A (gene that encodes p21) levels in Daoy cells and neurospheres. In Daoy cells, the upregulation of CDKN1A was accompanied by an increase in p21 protein levels. p21 is the target of several epigenetic regulations [31]. HDAC inhibition by SAHA demonstrated accumulation of acetylated H3 and H4 on the promoter region of p21 [32]. In addition, HDAC1 directly binds to the Sp1 site at the CDKN1A promoter region, preventing transcription [33]. VPA targets HDAC1 activity thereby it is likely that VPA disrupts HDAC1 repression of CDKN1A and

enables the increase of p21 levels. Hence, upregulation of p21 was followed by an increase in G1 arrest in Daoy cells, indicating that VPA arrests the cell cycle progression by modulation of cell cycle regulators. Even though our data is limited to CDKN1A levels in Daoy CSC, it is probable that VPA also promotes cell cycle arrest in this cellular context.

VPA modulation of p21 and cell cycle progression seems to be dependent on MB molecular subgroups. Daoy represents MB SHH whereas D283 is a group 3/group 4 cell line. Group 3 tumors frequently present amplification on the MYC gene. Although D283 does not have MYC amplification, D283 presents high levels of MYC similar to MYC amplificated MB cell lines [34-35]. MYC binds to the promoter region of CDKN1A and represses transcription [24-25]. Li and colleagues show that a long time of VPA exposure is necessary to reduce MYC expression in D283 in comparison to Daoy cells [18]. Here, we confirm that D283 cells have higher levels of MYC in comparison to Daoy cells and VPA was only able to reduce MYC levels in Daoy cells. Therefore, it is possible that 48 hours of exposure time was not sufficient to reduce MYC levels and thereby increase p21 levels in D283 cells. However, in the CSC context, VPA was capable of decreasing MYC in D283 and Daoy neurospheres, suggesting that CDKN1A increase in MB CSC can be associated with downregulation of MYC. Recent studies indicate that MYC activity can alter the epigenetic landscape, promoting a cellular reprogramming that favors a stem cell-like state [36-37]. Hence, our results indicate that VPA's ability to alter MYC signaling not only changes cycle progression but also contributes to a more differentiated state in MB CSC.

Induction of differentiation is an important strategy to reduce tumorigenicity, especially in the CSC context. Therapies that promote differentiation enable cancer cells with high malignancy to differentiate to a state of low tumorigenicity, increasing survival rates [38]. Cheng and colleagues demonstrated that induction of a neuronal differentiation process in glioma stem cells causes cell cycle arrest and inhibits proliferation [39]. Recent studies indicate that epigenetic modulators are essential to maintain the balance between differentiated and undifferentiated states [40]. HDAC inhibitors VPA, TSA, and NaB induces neuronal differentiation in progenitor cells [41]. In MB, previous studies indicate that NaB promotes differentiation and reduces stemness genes [22, 42]. Here, we found that the VPA antiproliferative effect on MB cells and CSC was followed by an increase in differentiation markers (Fig. 4). Upregulation of TUBB3, ENO2, and RBFOX3 was accompanied by morphologic changes similar to neuron-like in MB cells, indicating that

VPA promotes neuronal differentiation. Cheng and colleagues demonstrate that once MB cells differentiate, the cells lose their proliferative capacity and tumorigenic potential, and this process is crucially related to epigenetic modulation [43]. Hence, the neuronal differentiation induced by VPA in MB cells and CSC could indicate that it decreases MB malignancy.

VPA modulation seems to have an important role in defining the state of differentiation on MB, so we explore the SOX2 role in MB stemness. SOX2 is associated with self-renewal, pluripotency, and neuronal differentiation in embryonic stem cells [44-45]. In MB, recent studies indicate that SOX2 expression is crucial to MB development, especially in SHH molecular subgroup [46]. SHH MB tumors frequently harbor mutations that activate SHH signaling pathway. GLI1/2 are downstream factors of SHH and positively regulate SOX2 by binding to its promoter, promoting self-renewal and tumorigenesis [47]. In addition, SOX2-positive cells show lower sensitivity to chemotherapy agents, thereby contributing to tumor resistance and relapse [48]. Therefore, it was not surprising that SOX2 was upregulated in MB CSC in comparison to MB cells (Fig 1). VPA was able to reduce SOX2 transcriptional levels only in Daoy cells, indicating that the stemness pathways that maintain CSC differ according to MB molecular subgroup. In Daoy neurospheres, SOX2 levels were reduced by VPA, suggesting that VPA impairs CSC in SHH tumors through SOX2 expression. Moreover, VPA decreases Nestin transcriptional levels, which is a progenitor marker regulated by SOX2 [49], suggesting that VPA not only reduces SOX2 expression but also disrupts signaling pathways downstream of SOX2. VPA modulation of SOX2 could be related to p21 expression. Marqués-Torrejón and colleagues show that in neural stem cells p21 binds to SOX2 enhancer region, suppressing its transcription [50]. We found that VPA enhances CDKN1A expression in Daoy cells and neurospheres, reinforcing the idea of p21-SOX2 regulation in SHH MB. SOX2 is also susceptible to epigenetic regulation. Kidder and colleagues show that HDAC1 is a positive regulator of SOX2 in embryonic stem cells [51]. HDAC1 is a target of VPA modulation, thereby it is possible that VPA could influence HDAC1 regulation of SOX2.

So far, our results indicate that VPA has different mechanisms depending on the MB molecular subgroup. To further explore that idea, we choose to better understand the contribution of Notch signaling pathway. Notch signaling is known to promote proliferation by inhibiting neuronal differentiation and maintaining cells in a neural stem cell state [52].

In MB, Notch inhibition resulted in reduced CD133+ subpopulation and an increase in differentiation [53]. Here, we found the MB enriched CSC population has higher levels of Notch1 in comparison to MB cells (Fig. 1). VPA was able to reduce transcriptional levels of Notch1 and Hes1 only in D283 cells, suggesting that VPA modulates Notch1 specifically in the group 3/4 context. In D283 neurospheres, VPA was also capable of downregulating Notch1 and Hes1. Recent studies indicate that Notch1 signaling regulates self-renewal and metastasis in Group 3 MB [26]. Hence, VPA's abilities to reduce Notch1 levels or Notch1 + subpopulation, and the increase in differentiation suggest that VPA has therapeutic potential against Group 3 MB CSC. It's not yet clear how HDAC inhibitors regulate Notch1 expression but is believed to be related to modulation of the chromatin state [54].

In conclusion, our results indicate that VPA can induce MB to a state of low tumorigenicity by reducing MB viability, increasing differentiation, and downregulating stemness pathways. VPA effect seems to depend on the MB molecular subgroup. In SHH MB, VPA modulates the MYC-P21-SOX2 axis, whereas in Group 3 VPA regulates the Notch1 pathway. Therefore, our results provide evidence indicating that VPA inhibits cells with high malignant traits and should be further investigated with different cellular models such as *chemo resistant* cells and metastatic animal models.

References

1. Orr BA. (2020) Pathology, diagnostics, and classification of medulloblastoma. Brain Pathol 30: 664-678.

2. Zou H, Poore B, Broniscer A, Pollack IF, Hu B. (2020) Molecular Heterogeneity and Cellular Diversity: Implications for Precision Treatment in Medulloblastoma. Cancers (Basel) 12.

3. Juraschka K, Taylor MD. (2019) Medulloblastoma in the age of molecular subgroups: a review. J Neurosurg Pediatr 24: 353-363.

4. Taylor MD, et al. (2012) Molecular subgroups of medulloblastoma: the current consensus. Acta Neuropathol 123: 465-472.

5. Louis DN, et al. (2016) The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. Acta Neuropathol 131: 803-820.

6. Hill RM, et al. (2020) Time, pattern, and outcome of medulloblastoma relapse and their association with tumour biology at diagnosis and therapy: a multicentre cohort study. Lancet Child Adolesc Health 4: 865-874.

7. Beck B, Blanpain C. (2013) Unravelling cancer stem cell potential. Nat Rev Cancer 13: 727-738.

8. Rizzo S, et al. (2011) Ovarian cancer stem cell-like side populations are enriched following chemotherapy and overexpress EZH2. Mol Cancer Ther 10: 325-335.

9. Persson AI, Weiss WA. (2009) The side story of stem-like glioma cells. Cell Stem Cell 4: 191-192.

10. Moore N, Lyle S. (2011) Quiescent, slow-cycling stem cell populations in cancer: a review of the evidence and discussion of significance. J Oncol 2011.

11. Hemmati HD, et al. (2003) Cancerous stem cells can arise from pediatric brain tumors. Proc Natl Acad Sci U S A 100: 15178-15183.

12. Singh SK, et al. (2004) Identification of human brain tumour initiating cells. Nature 432: 396-401.

13. Huether R, et al. (2014) The landscape of somatic mutations in epigenetic regulators across 1,000 paediatric cancer genomes. Nat Commun 5: 3630.

14. Jagani Z, et al. (2010) Loss of the tumor suppressor Snf5 leads to aberrant activation of the Hedgehog-Gli pathway. Nat Med 16: 1429-1433.

15. Oleksiewicz U, et al. (2017) TRIM28 and Interacting KRAB-ZNFs Control Self-Renewal of Human Pluripotent Stem Cells through Epigenetic Repression of Pro-differentiation Genes. Stem Cell Reports 9: 2065-2080.

16. Wainwright EN, Scaffidi P. (2017) Epigenetics and Cancer Stem Cells: Unleashing, Hijacking, and Restricting Cellular Plasticity. Trends Cancer 3: 372-386.

17. Chateauvieux S, Morceau F, Dicato M, Diederich M. (2010) Molecular and therapeutic potential and toxicity of valproic acid. J Biomed Biotechnol 2010.

18. Li XN, et al. (2005) Valproic acid induces growth arrest, apoptosis, and senescence in medulloblastomas by increasing histone hyperacetylation and regulating expression of p21Cip1, CDK4, and CMYC. Mol Cancer Ther 4: 1912-1922.

19. Alvarez AA, Field M, Bushnev S, Longo MS, Sugaya K. (2015) The effects of histone deacetylase inhibitors on glioblastoma-derived stem cells. J Mol Neurosci 55: 7-20.

20. Riva G, et al. (2018) Valproic Acid Inhibits Proliferation and Reduces Invasiveness in Glioma Stem Cells Through Wnt/β Catenin Signalling Activation. Genes (Basel) 9.

21. S Franco S, et al. (2016) In vitro models of cancer stem cells and clinical applications. BMC Cancer 16: 738.

22. Nör C, et al. (2013) The histone deacetylase inhibitor sodium butyrate promotes cell death and differentiation and reduces neurosphere formation in human medulloblastoma cells. Mol Neurobiol 48: 533-543.

23. Singec I, et al. (2006) Defining the actual sensitivity and specificity of the neurosphere assay in stem cell biology. Nat Methods 3: 801-806.

24. Claassen GF, Hann SR. (2000) A role for transcriptional repression of p21CIP1 by c-Myc in overcoming transforming growth factor beta -induced cell-cycle arrest. Proc Natl Acad Sci U S A 97: 9498-9503.

25. Gartel AL, et al. (2001) Myc represses the p21(WAF1/CIP1) promoter and interacts with Sp1/Sp3. Proc Natl Acad Sci U S A 98: 4510-4515.

26. Kahn SA, et al. (2018) Notch1 regulates the initiation of metastasis and self-renewal of Group 3 medulloblastoma. Nat Commun 9: 4121.

27. Liu N, Li S, Wu N, Cho KS. (2017) Acetylation and deacetylation in cancer stem-like cells. Oncotarget 8: 89315-89325.

28. Shu Q, et al. (2006) Valproic Acid prolongs survival time of severe combined immunodeficient mice bearing intracerebellar orthotopic medulloblastoma xenografts. Clin Cancer Res 12: 4687-4694.

29. Marchion DC, Bicaku E, Daud AI, Sullivan DM, Munster PN. (2005) Valproic acid alters chromatin structure by regulation of chromatin modulation proteins. Cancer Res 65: 3815-3822.

30. Abbas T, Dutta A. (2009) p21 in cancer: intricate networks and multiple activities. Nat Rev Cancer 9: 400-414.

31. Ocker M, Bitar SA, Monteiro AC, Gali-Muhtasib H, Schneider-Stock R. (2019) Epigenetic Regulation of p21. Cancers (Basel) 11.

32. Richon VM, Sandhoff TW, Rifkind RA, Marks PA. (2000) Histone deacetylase inhibitor selectively induces p21WAF1 expression and gene-associated histone acetylation. Proc Natl Acad Sci U S A 97: 10014-10019.

33. Zupkovitz G, et al. (2010) The cyclin-dependent kinase inhibitor p21 is a crucial target for histone deacetylase 1 as a regulator of cellular proliferation. Mol Cell Biol 30: 1171-1181.

34. Bigner SH, Friedman HS, Vogelstein B, Oakes WJ, Bigner DD. (1990) Amplification of the c-myc gene in human medulloblastoma cell lines and xenografts. Cancer Res 50: 2347-2350.

35. Bruggers CS, et al. (1998) Expression of the c-Myc protein in childhood medulloblastoma. J Pediatr Hematol Oncol 20: 18-25.

36. Poli V, et al. (2018) MYC-driven epigenetic reprogramming favors the onset of tumorigenesis by inducing a stem cell-like state. Nat Commun 9: 1024.

37. Kozono D, et al. (2015) Dynamic epigenetic regulation of glioblastoma tumorigenicity through LSD1 modulation of MYC expression. Proc Natl Acad Sci U S A 112: E4055-4064.

38. Matthay KK, et al. (2009) Long-term results for children with high-risk neuroblastoma treated on a randomized trial of myeloablative therapy followed by 13-cis-retinoic acid: a children's oncology group study. J Clin Oncol 27: 1007-1013.

39. Cheng X, et al. (2019) Inhibition of Glioma Development by ASCL1-Mediated Direct Neuronal Reprogramming. Cells 8.

40. Zhang Z, et al. (2017) Similarity in gene-regulatory networks suggests that cancer cells share characteristics of embryonic neural cells. J Biol Chem 292: 12842-12859.

41. Hsieh J, Nakashima K, Kuwabara T, Mejia E, Gage FH. (2004) Histone deacetylase inhibition-mediated neuronal differentiation of multipotent adult neural progenitor cells. Proc Natl Acad Sci U S A 101: 16659-16664.

42. da Cunha Jaeger M, et al. (2020) HDAC and MAPK/ERK Inhibitors Cooperate To Reduce Viability and Stemness in Medulloblastoma. J Mol Neurosci 70: 981-992.

43. Cheng Y, et al. (2020) NeuroD1 Dictates Tumor Cell Differentiation in Medulloblastoma. Cell Rep 31: 107782.

44. Masui S, et al. (2007) Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. Nat Cell Biol 9: 625-635.

45. Thomson M, et al. (2011) Pluripotency factors in embryonic stem cells regulate differentiation into germ layers. Cell 145: 875-889.

46. Ahlfeld J, et al. (2013) Sox2 requirement in sonic hedgehog-associated medulloblastoma. Cancer Res 73: 3796-3807.

47. Santini R, et al. (2014) SOX2 regulates self-renewal and tumorigenicity of human melanoma-initiating cells. Oncogene 33: 4697-4708.

48. Vanner RJ, et al. (2014) Quiescent sox2(+) cells drive hierarchical growth and relapse in sonic hedgehog subgroup medulloblastoma. Cancer Cell 26: 33-47.

49. Tanaka S, et al. (2004) Interplay of SOX and POU factors in regulation of the Nestin gene in neural primordial cells. Mol Cell Biol 24: 8834-8846.

50. Marqués-Torrejón M, et al. (2013) Cyclin-dependent kinase inhibitor p21 controls adult neural stem cell expansion by regulating Sox2 gene expression. Cell Stem Cell 12: 88-100.

51. Kidder BL, Palmer S. (2012) HDAC1 regulates pluripotency and lineage specific transcriptional networks in embryonic and trophoblast stem cells. Nucleic Acids Res 40: 2925-2939.

52. Gaiano N, Fishell G. (2002) The role of notch in promoting glial and neural stem cell fates. Annu Rev Neurosci 25: 471-490.

53. Fan X, et al. (2006) Notch pathway inhibition depletes stem-like cells and blocks engraftment in embryonal brain tumors. Cancer Res 66: 7445-7452.

54. Schwanbeck R. (2015) The role of epigenetic mechanisms in Notch signaling during development. J Cell Physiol 230: 969-981.

DISCUSSÃO

As neoplasias pediátricas de sistema nervoso apresentam um dos principais desafios para o tratamento oncológico. Isto porque, tumores como MB, NB e pGM possuem elevadas taxas de resistência a tratamentos, formação de metástase e recidiva tumoral (JOHNSEN *et al*., 2019) (NORTHCOTT *et al*., 2019) (FUNAKOSHI *et al*., 2021). A alta capacidade tumorigênica e baixa sensibilidade aos tratamentos pode ser explicada pela presença de uma subpopulação de células, as células tronco tumorais (CTT) (ARAVINDAN *et al*., 2019). Um dos principais mecanismos envolvidos no estabelecimento e manutenção de um estado tronco tumoral são as modificações epigenéticas. Estudos sugerem que alterações no perfil epigenético permitem a reprogramação celular que contribuem para a ativação de vias de *stemness* (JAGANI et al., 2010) (OLEKSIEWICZ *et al*., 2017). Além disso, a escolha entre um estado diferenciado ou indiferenciado pode ser controlada por alterações no epigenoma (WAINWRIGHT & SCAFFIDI, 2017).

No capítulo I demonstramos que mecanismos epigenéticos como modificações em histonas, metilação do DNA, remodeladores da cromatina e regulação por microRNAs estão envolvidos na formação e manutenção de células tronco tumorais de tumores pediátricos do sistema nervoso. Além disso, foi possível perceber que existe uma dificuldade de transpor a modulação de mecanismos epigenéticos realizados em estudos de pesquisa básica para estudos clínicos. Neste sentido, o VPA, um modulador epigenético, apresenta vantagem por ser um fármaco considerado seguro para pacientes pediátricos, facilitando seu reposicionamento. Estudos clínicos com pacientes pediátricos utilizando VPA indicam que doses de VPA são bem toleradas e apresentam respostas clínicas promissoras no tratamento de tumores pediátricos de sistema nervoso (MASOUDI *et al*., 2008) (SU *et al*., 2011) (SU *et al*., 2020). É importante ressaltar que estudos clínicos em pacientes pediátricos abrangem tumores recorrentes e recidivos, assim, seus resultados são relevantes no contexto de células tronco tumorais.

VPA atua inibindo a ação de histonas deacetilases (HDAC) e, consequentemente, alterando a regulação da transcrição gênica. Um dos mecanismos regulados por HDAC é a progressão do ciclo celular, considerado um passo indispensável para proliferação e crescimento tumoral. HDAC são capazes de controlar a progressão do ciclo celular ao modular reguladores desse processo, como por exemplo: p53 (proteína de tumor p53), pRB (proteína retinoblastoma), p21 (inibidor de quinase dependente de ciclina 1) e p27 (inibidor

de quinase dependente de ciclina 1B) (TELES & SETO, 2011). Além disso, um dos principais mecanismos em comum entre inibidores de HDAC (HDACi) é a capacidade de alterar a progressão do ciclo celular (BOLDEN *et al*., 2006). Em NB, HDACi como Butirato de sódio (NaB), Tricostatina A (TSA) e VPA causam acúmulo de células em G0/G1 ou G2/M promovendo parada na progressão do ciclo celular (MÜHLETHALER-MOTTET *et al*., 2008) (GU *et al*., 2012). Estudos em MB utilizando Dacinostat e Quisinostat sugerem que inibir HDACs reduz a proliferação por meio da parada de ciclo celular (ZHANG *et al*., 2019).

Nosso estudo, apresentado no capítulo II, também demonstrou que o VPA foi capaz de causar parada na progressão do ciclo além de modular a expressão de p21 em MB. HDACi são capazes de regular p21 por causarem aumento de H3 e H4 acetiladas em regiões próximas ao promotor de p21, permitindo um aumento na transcrição deste gene que, por sua vez, regula a progressão do ciclo celular (RICHON *et al*., 2000). Sabe-se que a regulação das fases do ciclo tem suma importância na proliferação das células de câncer, mas a progressão do ciclo também está relacionada ao processo de diferenciação celular. Estudos indicam que a indução da diferenciação é dependente das fases do ciclo (PAUKLIN & VALLIER, 2014) (HARDWICK *et al*., 2015) e, dessa forma, a capacidade de HDACi de regular a progressão do ciclo também implica que estes inibidores são capazes de controlar o processo de diferenciação celular.

A diferenciação celular é um processo que permite que células tumorais adquiram um estado de menor tumorigenicidade, visto que células diferenciadas perdem a capacidade proliferativa e apresentam diminuição de traços de malignidade (CHENG *et al*., 2020). Estudos recentes indicam que a maquinaria epigenética é um fator chave para decisão entre um estado indiferenciado e diferenciado de uma célula (ZHANG *et al*., 2017). No capítulo I, foram elencados diferentes inibidores de HDAC capazes de causar aumento em marcadores de diferenciação neural e alterações morfológicas relacionadas à aquisição de um estado mais diferenciado em células de MB, NB e pGM. Assim, é sugerido que em tumores pediátricos de sistema nervoso, a redução da tumorigenicidade causada por HDACi está extremamente relacionada à indução de diferenciação. Nossos resultados em MB utilizando VPA reforçam esta premissa, visto que VPA foi capaz de aumentar marcadores de diferenciação neural, além de reduzir a viabilidade de células e CTT de MB.

Moduladores epigenéticos também são capazes de causar uma reprogramação celular que limita a sinalização de vias de *stemness*, diminuindo os traços característicos de células tronco tumorais. De acordo com o capítulo I, os genes de stemness CD133, SOX2, BMI1, NANOG e Nestin são os principais alvos de inibidores de HDAC em tumores pediátricos de sistema nervoso. Apesar da modulação de vias de *stemness* ser altamente dependente do contexto celular ou tumoral, é possível notarmos que um denominador em comum é que a modulação de genes de stemness está associada a redução na viabilidade e proliferação celular, declínio nas taxas de formação de CTT, e diminuição do crescimento *in vivo* (STOCKHAUSEN *et al*., 2005) (ZHENG *et al*., 2013) (ANASTAS *et al*., 2019) (DA CUNHA JAEGER *et al*., 2020). Os resultados experimentais do capítulo II, demonstram que, em MB, a redução na viabilidade e indução de diferenciação neural causadas por VPA podem ser também relacionadas a regulação dos genes de *stemness* SOX2, Nestina e Notch1 em células e CTT de MB. Ainda não foi possível definir se moduladores epigenéticos induzem diferenciação e este processo controla a expressão de genes de *stemness* ou se a modulação epigenética tem como alvo principal a regulação de genes de *stemness* e, por consequência, ocorre a indução de um estado mais diferenciado das células. Apesar do mecanismo específico de atuação de moduladores epigenéticos não estar claro, os resultados obtidos no capítulo I e II sugerem que inibidores epigenéticos, em especial aqueles que alteraram a acetilação de histonas, são potenciais terapias para tumores pediátricos de sistema nervoso visto que são capazes de reduzir a tumorigenicidade por alterar o estado da cromatina e permitir o estabelecimento de um perfil mais diferenciado nas células.

CONCLUSÃO

Nas últimas décadas os casos de neuroblastoma, meduloblastoma e glioma pediátricos têm aumentado e a principal característica comum entre estes tumores pediátricos do sistema nervoso são alterações epigenéticas, além das altas taxas de mortalidade e morbilidade. Logo, o uso de inibidores de HDAC como VPA tem se mostrado uma potencial estratégia terapêutica, principalmente por atingir células tronco tumorais, as quais são identificadas como responsáveis pelo aparecimento de metástases e recidiva tumoral. Dessa forma, concluímos os resultados *in vitro* sugerem que estudos que melhor caracterizem o

efeito de VPA, especialmente em tumores pediátricos do sistema nervoso com alta malignidade, devem ser continuados.

REFERÊNCIAS

ABBALLE, L.; MIELE, E. Epigenetic modulators for brain cancer stem cells: Implications for anticancer treatment. World J Stem Cells, 13, n. 7, p. 670-684, Jul 26 2021.

AL-HAJJ, M.; WICHA, M. S.; BENITO-HERNANDEZ, A.; MORRISON, S. J. et al. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A, 100, n. 7, p. 3983-3988, Apr 01 2003.

ALVAREZ, A. A.; FIELD, M.; BUSHNEV, S.; LONGO, M. S. et al. The effects of histone deacetylase inhibitors on glioblastoma-derived stem cells. J Mol Neurosci, 55, n. 1, p. 7-20, Jan 2015.

ANASTAS, J. N.; ZEE, B. M.; KALIN, J. H.; KIM, M. et al. Re-programing Chromatin with a Bifunctional LSD1/HDAC Inhibitor Induces Therapeutic Differentiation in DIPG. Cancer Cell, 36, n. 5, p. 528-544.e510, 11 11 2019.

ARAVINDAN, N.; JAIN, D.; SOMASUNDARAM, D. B.; HERMAN, T. S. et al. Cancer stem cells in neuroblastoma therapy resistance. Cancer Drug Resist, 2, p. 948-967, Dec 19 2019.

AYOB, A. Z.; RAMASAMY, T. S. Cancer stem cells as key drivers of tumour progression. J Biomed Sci, 25, n. 1, p. 20, Mar 06 2018.

BAO, B.; AHMAD, A.; AZMI, A. S.; ALI, S. et al. Overview of cancer stem cells (CSCs) and mechanisms of their regulation: implications for cancer therapy. Curr Protoc Pharmacol, Chapter 14, p. Unit 14.25, Jun 2013.

BECK, B.; BLANPAIN, C. Unravelling cancer stem cell potential. Nat Rev Cancer, 13, n. 10, p. 727-738, 10 2013.

BLEAU, A. M.; HAMBARDZUMYAN, D.; OZAWA, T.; FOMCHENKO, E. I. et al. PTEN/PI3K/Akt pathway regulates the side population phenotype and ABCG2 activity in glioma tumor stem-like cells. Cell Stem Cell, 4, n. 3, p. 226-235, Mar 06 2009.

BOLDEN, J. E.; PEART, M. J.; JOHNSTONE, R. W. Anticancer activities of histone deacetylase inhibitors. Nat Rev Drug Discov, 5, n. 9, p. 769-784, Sep 2006.

BONNET, D.; DICK, J. E. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med, 3, n. 7, p. 730-737, Jul 1997.

BRADBURY, C. A.; KHANIM, F. L.; HAYDEN, R.; BUNCE, C. M. et al. Histone deacetylases in acute myeloid leukaemia show a distinctive pattern of expression that changes selectively in response to deacetylase inhibitors. Leukemia, 19, n. 10, p. 1751-1759, Oct 2005.

CABRERA, M. C.; HOLLINGSWORTH, R. E.; HURT, E. M. Cancer stem cell plasticity and tumor hierarchy. World J Stem Cells, 7, n. 1, p. 27-36, Jan 26 2015.

CAVALLI, F. M. G.; REMKE, M.; RAMPASEK, L.; PEACOCK, J. et al. Intertumoral Heterogeneity within Medulloblastoma Subgroups. Cancer Cell, 31, n. 6, p. 737-754.e736, 06 12 2017.

CHATEAUVIEUX, S.; MORCEAU, F.; DICATO, M.; DIEDERICH, M. Molecular and therapeutic potential and toxicity of valproic acid. J Biomed Biotechnol, 2010, 2010.

CHENG, Y.; LIAO, S.; XU, G.; HU, J. et al. NeuroD1 Dictates Tumor Cell Differentiation in Medulloblastoma. Cell Rep, 31, n. 12, p. 107782, 06 23 2020.

CHEVIGNARD, M.; CÂMARA-COSTA, H.; DOZ, F.; DELLATOLAS, G. Core deficits and quality of survival after childhood medulloblastoma: a review. Neurooncol Pract, 4, n. 2, p. 82-97, Jun 2017.

CORRÒ, C.; MOCH, H. Biomarker discovery for renal cancer stem cells. J Pathol Clin Res, 4, n. 1, p. 3-18, Jan 2018.

DA CUNHA JAEGER, M.; GHISLENI, E. C.; CARDOSO, P. S.; SINIGLAGLIA, M. et al. HDAC and MAPK/ERK Inhibitors Cooperate To Reduce Viability and Stemness in Medulloblastoma. J Mol Neurosci, 70, n. 6, p. 981-992, Jun 2020.

DAWSON, M. A.; KOUZARIDES, T. Cancer epigenetics: from mechanism to therapy. Cell, 150, n. 1, p. 12-27, Jul 06 2012.

DIEHN, M.; CHO, R. W.; LOBO, N. A.; KALISKY, T. et al. Association of reactive oxygen species levels and radioresistance in cancer stem cells. Nature, 458, n. 7239, p. 780-783, Apr 09 2009.

FAN, X.; EBERHART, C. G. Medulloblastoma stem cells. J Clin Oncol, 26, n. 17, p. 2821-2827, Jun 10 2008.

FATTET, S.; HABERLER, C.; LEGOIX, P.; VARLET, P. et al. Beta-catenin status in paediatric medulloblastomas: correlation of immunohistochemical expression with mutational status, genetic profiles, and clinical characteristics. J Pathol, 218, n. 1, p. 86-94, May 2009.

FUNAKOSHI, Y.; HATA, N.; KUGA, D.; HATAE, R. et al. Pediatric Glioma: An Update of Diagnosis, Biology, and Treatment. Cancers (Basel), 13, n. 4, Feb 12 2021.

GEAN, P. W.; HUANG, C. C.; HUNG, C. R.; TSAI, J. J. Valproic acid suppresses the synaptic response mediated by the NMDA receptors in rat amygdalar slices. Brain Res Bull, 33, n. 3, p. 333-336, 1994.

GERDES, M. J.; SOOD, A.; SEVINSKY, C.; PRIS, A. D. et al. Emerging understanding of multiscale tumor heterogeneity. Front Oncol, 4, p. 366, 2014.

GIBSON, P.; TONG, Y.; ROBINSON, G.; THOMPSON, M. C. et al. Subtypes of medulloblastoma have distinct developmental origins. Nature, 468, n. 7327, p. 1095-1099, Dec 23 2010.

GRÖBNER, S. N.; WORST, B. C.; WEISCHENFELDT, J.; BUCHHALTER, I. et al. The landscape of genomic alterations across childhood cancers. Nature, 555, n. 7696, p. 321-327, 03 15 2018.

GU, S.; TIAN, Y.; CHLENSKI, A.; SALWEN, H. R. et al. Valproic acid shows a potent antitumor effect with alteration of DNA methylation in neuroblastoma. Anticancer Drugs, 23, n. 10, p. 1054-1066, Nov 2012.

GÖTTLICHER, M.; MINUCCI, S.; ZHU, P.; KRÄMER, O. H. et al. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. EMBO J, 20, n. 24, p. 6969-6978, Dec 17 2001.

HANAHAN, D.; WEINBERG, R. A. Hallmarks of cancer: the next generation. Cell, 144, n. 5, p. 646-674, Mar 04 2011.

HARDWICK, L. J.; ALI, F. R.; AZZARELLI, R.; PHILPOTT, A. Cell cycle regulation of proliferation versus differentiation in the central nervous system. Cell Tissue Res, 359, n. 1, p. 187-200, Jan 2015.

HEMMATI, H. D.; NAKANO, I.; LAZAREFF, J. A.; MASTERMAN-SMITH, M. et al. Cancerous stem cells can arise from pediatric brain tumors. Proc Natl Acad Sci U S A, 100, n. 25, p. 15178-15183, Dec 09 2003.

HUETHER, R.; DONG, L.; CHEN, X.; WU, G. et al. The landscape of somatic mutations in epigenetic regulators across 1,000 paediatric cancer genomes. Nat Commun, 5, p. 3630, Apr 08 2014.

JAGANI, Z.; MORA-BLANCO, E. L.; SANSAM, C. G.; MCKENNA, E. S. et al. Loss of the tumor suppressor Snf5 leads to aberrant activation of the Hedgehog-Gli pathway. Nat Med, 16, n. 12, p. 1429-1433, Dec 2010.

JOHNSEN, J. I.; DYBERG, C.; WICKSTRÖM, M. Neuroblastoma-A Neural Crest Derived Embryonal Malignancy. Front Mol Neurosci, 12, p. 9, 2019.

JURASCHKA, K.; TAYLOR, M. D. Medulloblastoma in the age of molecular subgroups: a review. J Neurosurg Pediatr, 24, n. 4, p. 353-363, 10 01 2019.

KOOL, M.; KORSHUNOV, A.; REMKE, M.; JONES, D. T. et al. Molecular subgroups of medulloblastoma: an international meta-analysis of transcriptome, genetic aberrations, and clinical data of WNT, SHH, Group 3, and Group 4 medulloblastomas. Acta Neuropathol, 123, n. 4, p. 473-484, Apr 2012.

KUMAR, V.; MCGUIRE, T.; COULTER, D. W.; SHARP, J. G. et al. Challenges and Recent Advances in Medulloblastoma Therapy. Trends Pharmacol Sci, 38, n. 12, p. 1061-1084, 12 2017.

LAPIDOT, T.; SIRARD, C.; VORMOOR, J.; MURDOCH, B. et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature, 367, n. 6464, p. 645-648, Feb 17 1994.

LI, X. N.; SHU, Q.; SU, J. M.; PERLAKY, L. et al. Valproic acid induces growth arrest, apoptosis, and senescence in medulloblastomas by increasing histone hyperacetylation and regulating expression of p21Cip1, CDK4, and CMYC. Mol Cancer Ther, 4, n. 12, p. 1912-1922, Dec 2005.

LIU, N.; LI, S.; WU, N.; CHO, K. S. Acetylation and deacetylation in cancer stem-like cells. Oncotarget, 8, n. 51, p. 89315-89325, Oct 24 2017.

MARMORSTEIN, R.; ZHOU, M. M. Writers and readers of histone acetylation: structure, mechanism, and inhibition. Cold Spring Harb Perspect Biol, 6, n. 7, p. a018762, Jul 01 2014.

MASOUDI, A.; ELOPRE, M.; AMINI, E.; NAGEL, M. E. et al. Influence of valproic acid on outcome of high-grade gliomas in children. Anticancer Res, 28, n. 4C, p. 2437-2442, 2008 Jul-Aug 2008.

MESDJIAN, E.; CIESIELSKI, L.; VALLI, M.; BRUGUEROLLE, B. et al. Sodium valproate: kinetic profile and effects on GABA levels in various brain areas of the rat. Prog Neuropsychopharmacol Biol Psychiatry, 6, n. 3, p. 223-233, 1982.

MOORE, N.; LYLE, S. Quiescent, slow-cycling stem cell populations in cancer: a review of the evidence and discussion of significance. J Oncol, 2011, 2011.

MÜHLETHALER-MOTTET, A.; MEIER, R.; FLAHAUT, M.; BOURLOUD, K. B. et al. Complex molecular mechanisms cooperate to mediate histone deacetylase inhibitors anti-tumour activity in neuroblastoma cells. Mol Cancer, 7, p. 55, Jun 12 2008.

NGUYEN, L. V.; VANNER, R.; DIRKS, P.; EAVES, C. J. Cancer stem cells: an evolving concept. Nat Rev Cancer, 12, n. 2, p. 133-143, 01 12 2012.

NIGHTINGALE, K. P.; O'NEILL, L. P.; TURNER, B. M. Histone modifications: signalling receptors and potential elements of a heritable epigenetic code. Curr Opin Genet Dev, 16, n. 2, p. 125-136, Apr 2006.

NORTHCOTT, P. A.; BUCHHALTER, I.; MORRISSY, A. S.; HOVESTADT, V. et al. The whole-genome landscape of medulloblastoma subtypes. Nature, 547, n. 7663, p. 311-317, 07 19 2017.

NORTHCOTT, P. A.; NAKAHARA, Y.; WU, X.; FEUK, L. et al. Multiple recurrent genetic events converge on control of histone lysine methylation in medulloblastoma. Nat Genet, 41, n. 4, p. 465-472, Apr 2009.

NORTHCOTT, P. A.; ROBINSON, G. W.; KRATZ, C. P.; MABBOTT, D. J. et al. Medulloblastoma. Nat Rev Dis Primers, 5, n. 1, p. 11, 02 14 2019.

NORTHCOTT, P. A.; SHIH, D. J.; PEACOCK, J.; GARZIA, L. et al. Subgroup-specific structural variation across 1,000 medulloblastoma genomes. Nature, 488, n. 7409, p. 49-56, Aug 02 2012.

OLEKSIEWICZ, U.; GŁADYCH, M.; RAMAN, A. T.; HEYN, H. et al. TRIM28 and Interacting KRAB-ZNFs Control Self-Renewal of Human Pluripotent Stem Cells through Epigenetic Repression of Pro-differentiation Genes. Stem Cell Reports, 9, n. 6, p. 2065-2080, 12 12 2017.

ORR, B. A. Pathology, diagnostics, and classification of medulloblastoma. Brain Pathol, 30, n. 3, p. 664-678, 05 2020.

OSTROM, Q. T.; CIOFFI, G.; GITTLEMAN, H.; PATIL, N. et al. CBTRUS Statistical Report: Primary Brain and Other Central Nervous System Tumors Diagnosed in the United States in 2012-2016. Neuro Oncol, 21, n. Suppl 5, p. v1-v100, 11 01 2019.

PAUKLIN, S.; VALLIER, L. The Cell-Cycle State of Stem Cells Determines Cell Fate Propensity. Cell, 156, n. 6, p. 1338, Mar 13 2014.

PAWLIK, T. M.; KEYOMARSI, K. Role of cell cycle in mediating sensitivity to radiotherapy. Int J Radiat Oncol Biol Phys, 59, n. 4, p. 928-942, Jul 15 2004.

PHIEL, C. J.; ZHANG, F.; HUANG, E. Y.; GUENTHER, M. G. et al. Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. J Biol Chem, 276, n. 39, p. 36734-36741, Sep 28 2001.

PRASETYANTI, P. R.; MEDEMA, J. P. Intra-tumor heterogeneity from a cancer stem cell perspective. Mol Cancer, 16, n. 1, p. 41, 02 16 2017.

RAMASWAMY, V.; REMKE, M.; BOUFFET, E.; BAILEY, S. et al. Risk stratification of childhood medulloblastoma in the molecular era: the current consensus. Acta Neuropathol, 131, n. 6, p. 821-831, 06 2016.

RAMASWAMY, V.; REMKE, M.; BOUFFET, E.; FARIA, C. C. et al. Recurrence patterns across medulloblastoma subgroups: an integrated clinical and molecular analysis. Lancet Oncol, 14, n. 12, p. 1200-1207, Nov 2013.

RICH, J. N. Cancer stem cells: understanding tumor hierarchy and heterogeneity. Medicine (Baltimore), 95, n. 1 Suppl 1, p. S2-S7, Sep 2016.

RICHON, V. M.; SANDHOFF, T. W.; RIFKIND, R. A.; MARKS, P. A. Histone deacetylase inhibitor selectively induces p21WAF1 expression and gene-associated histone acetylation. Proc Natl Acad Sci U S A, 97, n. 18, p. 10014-10019, Aug 29 2000.

RIZZO, S.; HERSEY, J. M.; MELLOR, P.; DAI, W. et al. Ovarian cancer stem cell-like side populations are enriched following chemotherapy and overexpress EZH2. Mol Cancer Ther, 10, n. 2, p. 325-335, Feb 2011.

ROBINSON, G.; PARKER, M.; KRANENBURG, T. A.; LU, C. et al. Novel mutations target distinct subgroups of medulloblastoma. Nature, 488, n. 7409, p. 43-48, Aug 02 2012.

ROUSSEL, M. F.; STRIPAY, J. L. Epigenetic Drivers in Pediatric Medulloblastoma. Cerebellum, 17, n. 1, p. 28-36, 02 2018.

SHU, Q.; ANTALFFY, B.; SU, J. M.; ADESINA, A. et al. Valproic Acid prolongs survival time of severe combined immunodeficient mice bearing intracerebellar orthotopic medulloblastoma xenografts. Clin Cancer Res, 12, n. 15, p. 4687-4694, Aug 01 2006.

SIEGEL, R. L.; MILLER, K. D.; FUCHS, H. E.; JEMAL, A. Cancer statistics, 2022. CA Cancer J Clin, 72, n. 1, p. 7-33, 01 2022.

SINGH, B. N.; ZHANG, G.; HWA, Y. L.; LI, J. et al. Nonhistone protein acetylation as cancer therapy targets. Expert Rev Anticancer Ther, 10, n. 6, p. 935-954, Jun 2010.

SINGH, S. K.; CLARKE, I. D.; TERASAKI, M.; BONN, V. E. et al. Identification of a cancer stem cell in human brain tumors. Cancer Res, 63, n. 18, p. 5821-5828, Sep 15 2003.

STOCKHAUSEN, M. T.; SJÖLUND, J.; MANETOPOULOS, C.; AXELSON, H. Effects of the histone deacetylase inhibitor valproic acid on Notch signalling in human neuroblastoma cells. Br J Cancer, 92, n. 4, p. 751-759, Feb 28 2005.

STORCI, G.; SANSONE, P.; MARI, S.; D'UVA, G. et al. TNFalpha up-regulates SLUG via the NF-kappaB/HIF1alpha axis, which imparts breast cancer cells with a stem cell-like phenotype. J Cell Physiol, 225, n. 3, p. 682-691, Nov 2010.

SU, J. M.; LI, X. N.; THOMPSON, P.; OU, C. N. et al. Phase 1 study of valproic acid in pediatric patients with refractory solid or CNS tumors: a children's oncology group report. Clin Cancer Res, 17, n. 3, p. 589-597, Feb 01 2011.

SU, J. M.; MURRAY, J. C.; MCNALL-KNAPP, R. Y.; BOWERS, D. C. et al. A phase 2 study of valproic acid and radiation, followed by maintenance valproic acid and bevacizumab in children with newly diagnosed diffuse intrinsic pontine glioma or high-grade glioma. Pediatr Blood Cancer, 67, n. 6, p. e28283, 06 2020.

TAYLOR, M. D.; NORTHCOTT, P. A.; KORSHUNOV, A.; REMKE, M. et al. Molecular subgroups of medulloblastoma: the current consensus. Acta Neuropathol, 123, n. 4, p. 465-472, Apr 2012.

TELLES, E.; SETO, E. Modulation of cell cycle regulators by HDACs. Front Biosci (Schol Ed), 4, n. 3, p. 831-839, 01 01 2012.

THOMPSON, E. M.; HIELSCHER, T.; BOUFFET, E.; REMKE, M. et al. Prognostic value of medulloblastoma extent of resection after accounting for molecular subgroup: a retrospective integrated clinical and molecular analysis. Lancet Oncol, 17, n. 4, p. 484-495, 04 2016.

THOMPSON, M. C.; FULLER, C.; HOGG, T. L.; DALTON, J. et al. Genomics identifies medulloblastoma subgroups that are enriched for specific genetic alterations. J Clin Oncol, 24, n. 12, p. 1924-1931, Apr 20 2006.

TOH, T. B.; LIM, J. J.; CHOW, E. K. Epigenetics in cancer stem cells. Mol Cancer, 16, n. 1, p. 29, 02 01 2017.

VAN BREEMEN, M. S.; WILMS, E. B.; VECHT, C. J. Epilepsy in patients with brain tumours: epidemiology, mechanisms, and management. Lancet Neurol, 6, n. 5, p. 421-430, May 2007.

VANDONGEN, A. M.; VANERP, M. G.; VOSKUYL, R. A. Valproate reduces excitability by blockage of sodium and potassium conductance. Epilepsia, 27, n. 3, p. 177-182, 1986 May-Jun 1986.

WAINWRIGHT, E. N.; SCAFFIDI, P. Epigenetics and Cancer Stem Cells: Unleashing, Hijacking, and Restricting Cellular Plasticity. Trends Cancer, 3, n. 5, p. 372-386, 05 2017.

WOLFF, J. E.; KRAMM, C.; KORTMANN, R. D.; PIETSCH, T. et al. Valproic acid was well tolerated in heavily pretreated pediatric patients with high-grade glioma. J Neurooncol, 90, n. 3, p. 309-314, Dec 2008.

YANG, X. J.; SETO, E. HATs and HDACs: from structure, function and regulation to novel strategies for therapy and prevention. Oncogene, 26, n. 37, p. 5310-5318, Aug 13 2007.

ZHANG, S.; GONG, Z.; OLADIMEJI, P. O.; CURRIER, D. G. et al. A high-throughput screening identifies histone deacetylase inhibitors as therapeutic agents against medulloblastoma. Exp Hematol Oncol, 8, p. 30, 2019.

ZHANG, Z.; LEI, A.; XU, L.; CHEN, L. et al. Similarity in gene-regulatory networks suggests that cancer cells share characteristics of embryonic neural cells. J Biol Chem, 292, n. 31, p. 12842-12859, 08 04 2017.

ZHAO, S.; ALLIS, C. D.; WANG, G. G. The language of chromatin modification in human cancers. Nat Rev Cancer, 21, n. 7, p. 413-430, 07 2021.

ZHAO, Z.; SHILATIFARD, A. Epigenetic modifications of histones in cancer. Genome Biol, 20, n. 1, p. 245, 11 20 2019.

ZHENG, X.; NAIDITCH, J.; CZURYLO, M.; JIE, C. et al. Differential effect of long-term drug selection with doxorubicin and vorinostat on neuroblastoma cells with cancer stem cell characteristics. Cell Death Dis, 4, p. e740, Jul 25 2013.

APÊNDICE 1: CURRICULUM VITAE RESUMIDO

CURRICULUM VITÆ

Freire, N.H.

1. DADOS PESSOAIS
   Nome: Natália Hogetop Freire
   Nascida em Porto Alegre, RS, Brasil, em 03 de dezembro de 1996.
   Endereço profissional: Laboratório de Câncer e Neurobiologia, Centro de Pesquisa Experimental, Hospital de Clínicas de Porto Alegre Rua Ramiro Barcelos, 2350, Av. Protásio Alves, 211 - Santa Cecília, Porto Alegre - RS, 90035-903
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2. FORMAÇÃO:
   Bacharel em Biotecnologia pela Universidade Federal do Rio Grande do Sul (2016-2019).
   Trabalho de conclusão de curso: Inibição De G9a Como Um Potencial Alvo Terapêutico: Uma Avaliação Em Tumores Pediátricos. Orientador: Prof. Dr. Rafael Roesler. Coorientadora: Dra. Bárbara Kunzler Souza

   Mestrado em andamento em biologia molecular e celular (2020). Universidade Federal do Rio Grande do Sul, UFRGS, Brasil. Título: Epigenética em tumores pediátricos do sistema nervoso: efeito do ácido valpróico em células tronco de meduloblastoma. Orientador: Dr. Rafael Roesler. Coorientadora: Dra. Mariane da Cunha Jaeger.

3. ESTÁGIOS:
   2019/2: Estágio curricular sob orientação da prof. Dr. Úrsula Matte.

   Setembro de 2017 – dezembro de 2020: Iniciação científica. Bolsista CNPq de Iniciação Científica no Laboratório de Câncer e Neurobiologia, sob orientação da Prof. Dr. Rafael Roesler.

4. ARTIGOS COMPLETOS PUBLICADOS

   BUENDIA DUQUE, M.; PINHEIRO, K. V.; THOMAZ, A.; DA SILVA, C. A. et al. Combined Inhibition of HDAC and EGFR Reduces Viability and Proliferation and Enhances STAT3 mRNA Expression in Glioblastoma Cells. J Mol Neurosci, 68, n. 1, p. 49-57, May 2019.

   PINHEIRO, K. V.; THOMAZ, A.; SOUZA, B. K.; METCALFE, V. A. et al. Expression and pharmacological inhibition of TrkB and EGFR in glioblastoma. Mol Biol Rep, 47, n. 9, p. 6817-6828, Sep 2020.

SOUZA, B. K.; **FREIRE, N. H.;** JAEGER, M.; DE FARIAS, C. B. et al. EHMT2/G9a as an Epigenetic Target in Pediatric and Adult Brain Tumors. Int J Mol Sci, 22, n. 20, Oct 19 2021.

SOUZA, B. K.; **FREIRE, N. H.**; JAEGER, M.; DE FARIAS, C. B. et al. G9a/EHMT2 is a Potential Prognostic Biomarker and Molecular Target in SHH Medulloblastoma. Neuromolecular Med, Feb 03 2022.

5. RESUMOS E TRABALHOS APRESENTADOS EM CONGRESSOS

SOUZA, B. K.; **FREIRE, N. H.**; BRUNETTO, A.T; BRUNETTO, A.L.; GREGIANIN, L. J.; DE FARIAS, C. B; ROESLER, R. O papel funcional de G9a/EHMT2 na biologia de tumores sólidos pediátricos. XVI Congresso Brasileiro de Oncologia Pediatria, Foz do Iguaçu, Brasil, 2018

PINHEIRO, K. V.; **FREIRE, N. H**.; SOUZA, B. K.; THOMAZ, A.; ISOLAN, G. R.; BRUNETTO, A.T; DE FARIAS, C. B; ROESLER, R. Efeito do bloqueio seletivo de TRKB e EGFR em glioblastoma. XVI Congresso Brasileiro de Oncologia Pediatria, Foz do Iguaçu, Brasil, 2018

THOMAZ, A.; PINHEIRO, K. V.; SOUZA, B. K.; **FREIRE, N. H**.; JAEGER, M.; EVANGELISTA, M. C. J.; BRUNETTO, A.L; DE FARIAS, C. B; ROESLER, R. Efeitos biológicos do bloqueio seletivo de TrkB em modelos pré-clínicos de meduloblastoma. XVI Congresso Brasileiro de Oncologia Pediatria, Foz do Iguaçu, Brasil, 2018

FREIRE, N. H.; PINHEIRO, K. V.; THOMAZ, A.; SOUZA, B. K.; BRUNETTO, A.T; DE FARIAS, C. B; ROESLER, R. BDNF/TRKB em glioma: interações funcionais com EGFR. 39ª Semana Científica do HCPA, realizada no Hospital de Clínicas de Porto Alegre, 2019.

FREIRE, N. H.; SOUZA, B. K.; DE FARIAS, C. B; JAEGER, M.; ROESLER, R. Inibição de G9a reduz a proliferação em células de Sarcoma de Ewing. XXXI Congreso de la Sociedad Latinoamericana de Oncología Pediátrica, 2020.

BATTISTELLA, M.E.; **FREIRE, N. H.**; TOSON, B.; SOUZA, B. K.; BRUNETTO, A.T; JAEGER, M.; DE FARIAS, C. B; ROESLER, R. "Desvendando a biologia do Câncer pediátrico: avaliação do efeito do ácido retinóico em células tumorais de Sarcoma de Ewing. XXXI Congreso de la Sociedad Latinoamericana de Oncología Pediátrica, 2020.

FREIRE, N. H.; DOS SANTOS, B. A.; BRUNETTO, A.T; DE FARIAS, C. B; JAEGER, M.; ROESLER, R. Inibição Combinada de EGFR e TRKB em Meduloblastoma. 40ª Semana Científica do HCPA, realizada no Hospital de Clínicas de Porto Alegre, 2020.

FUKUOKA, B. D.; BRUNETTO, A.T; **FREIRE, N. H.**; TOSON, B.; BRUNETTO, A.L; DE FARIAS, C. B; JAEGER, M.; ROESLER, R. Inibição de TRKB em Câncer de Próstata. 40ª Semana Científica do HCPA, realizada no Hospital de Clínicas de Porto Alegre, 2020.

FREIRE, N. H.; JAEGER, M.; BRUNETTO, A.T; ROESLER, R. o Efeito do ácido valproico em células tronco tumorais de meduloblastoma humano. 41ª Semana Científica do HCPA, realizada no Hospital de Clínicas de Porto Alegre, 2021.

FREIRE, N. H.; JAEGER, M.; BRUNETTO, A.T; DE FARIAS, C. B; GREGIANIN, L. J.; ROESLER, R. o Efeito do ácido valproico em células tronco tumorais de meduloblastoma humano. Congresso Internacional do GRAACC, 2022.

JAEGER, M.; **FREIRE, N. H.**; BIZZI, J. W. J.; DE FARIAS, C. B.; ROESLER, R.; GREGIANIN, L. J. Efeito antitumoral de ácido valpróico em modelos in vitro de glioma pediátrico. Congresso Internacional do GRAACC, 2022.

BORGMANN, N. O.; FUKUOKA, B. D.; **FREIRE, N. H.**; ROESLER, R; DE FARIAS, C. B; JAEGER, M. Alteração de EGFR por Inibidores de HDAC em Sarcoma de Ewing. 42ª Semana Científica do HCPA, realizada no Hospital de Clínicas de Porto Alegre, 2022.

FREIRE, N. H.; JAEGER, M.; BRUNETTO, A.T; ROESLER, R. o Efeito do ácido valproico em células tronco tumorais de meduloblastoma humano. 42ª Semana Científica do HCPA, realizada no Hospital de Clínicas de Porto Alegre, 2022.