Translational Oncology 13 (2020) 100819

Contents lists available at ScienceDirect

Translational Oncology

journal homepage: www.elsevier.com/locate/tranon

Original research

The neural stem-cell marker *CD24* is specifically upregulated in IDH-mutant glioma

Patricia D.B. Tiburcio [a,b,1], Mary C. Locke [c], Srividya Bhaskara [b,c], Mahesh B. Chandrasekharan [c], L. Eric Huang [a,b,*]

[a] Department of Neurosurgery, Clinical Neurosciences Center, University of Utah, Salt Lake City, UT, USA
[b] Department of Oncological Sciences, Huntsman Cancer Institute, University of Utah, Salt Lake City, UT, USA
[c] Department of Radiation Oncology, Huntsman Cancer Institute, University of Utah, Salt Lake City, UT, USA

ARTICLE INFO

Article history:
Received 24 April 2020
Received in revised form 25 May 2020
Accepted 27 May 2020
Available online xxxx

ABSTRACT

Background: Malignant gliomas have disproportionally high morbidity and mortality. Heterozygous mutations in the isocitrate dehydrogenase 1 (*IDH1*) gene are most common in glioma, resulting in predominantly arginine to histidine substitution at codon 132. Because $IDH1^{R132H}$ requires a wild-type allele to produce (D)-2-hydroxyglutarate for epigenetic reprogramming, loss of $IDH1^{R132H}$ heterozygosity is associated with glioma progression in an *IDH1*-wildtype-like phenotype. Although previous studies have reported that transgenic $IDH1^{R132H}$ induces the expression of nestin—a neural stem-cell marker, the underlying mechanism remains unclear. Furthermore, this finding seems at odds with better outcome of $IDH1^{R132H}$ glioma because of a negative association of nestin with overall survival.
Methods: Gene expression was compared between $IDH1^{R132H}$-hemizygous and $IDH1^{R132H}$-heterozygous glioma cells under adherent and spheroid growth conditions. The results were validated for (D)-2-hydroxyglutarate responsiveness by pharmacologic agents, associations with DNA methylation by bioinformatic analysis, and associations with overall survival. Bisulfite DNA sequencing, chromatin immunoprecipitation, and pharmacological approach were used.
Findings: Neural stem-cell marker genes, including *CD44*, *NES*, and *PROM1*, are generally downregulated in IDH-mutant gliomas and $IDH1^{R132H}$-heterozygous spheroid growth compared respectively with IDH-wildtype gliomas and $IDH1^{R132H}$-hemizygous spheroid growth, in agreement with their negative associations with patient outcome. In contrast, *CD24* is specifically upregulated and apparently associated with better survival. *CD24* and *NES* expression respond differentially to alteration of (D)-2-hydroxyglutarate levels. *CD24* upregulation is associated with histone and DNA demethylation as opposed to hypermethylation in the downregulated genes.
Interpretation: The better outcome of IDH-mutant glioma is orchestrated exquisitely through epigenetic reprogramming that directs bidirectional expression of neural stem-cell marker genes.

Introduction

Gliomas represent 81% of primary brain malignancy and cause significant morbidity and mortality [1]. Whereas glioblastoma (WHO grade IV)—the most common and advanced form of glioma—has a 5-year survival of only 5.5%, WHO grade II and grade III (lower-grade) gliomas—owing to the inevitable recurrence and progression—contribute disproportionately to the high mortality and morbidity [2]. Despite the emergence of novel therapeutics including molecular targeting, the outcome remains dismal.

The human *IDH1* gene encodes the cytosolic isocitrate dehydrogenase that catalyzes the conversion of isocitrate and $NADP^+$ to 2-oxoglutarate (aka α-ketoglutarate) and NADPH. Hotspot heterozygous mutations in *IDH1* occur in >70% of the lower-grade gliomas and secondary glioblastomas, resulting predominantly in the substitution of arginine 132 with histidine [3]. The mutant enzyme $IDH1^{R132H}$ acquires a neomorphic activity that converts 2-oxoglutarate and NADPH further to (D)-2-hydroxyglutarate (D-2HG) [4,5], thereby leading to $NADP^+$/NADPH imbalance [6,7]. High concentrations of D-2HG inhibit 2-oxoglutarate-dependent histone demethylases and 5-methylcytosine hydroxylases, leading to hypermethylation of lysine residues in histones and CpG islands in DNA [8,9]. It is generally believed that the epigenetic reprogramming through histone and DNA hypermethylation recapitulates the glioma-CpG island methylator phenotype to block cell differentiation and drive IDH-mutant glioma development [9–11].

Interestingly, $IDH1^{R132H}$ neomorphic activity requires the expression of wild-type *IDH1*; $IDH1^{R132H}$ alone is insufficient to produce D-2HG [12–14]. As such, $IDH1^{R132H}$-hemizygous glioma cells (resulting from the loss of

* Address all correspondence to: L. Eric Huang, Department of Neurosurgery, Clinical Neurosciences Center, 175 North Medical Drive East, Salt Lake City, UT 84132, USA.
E-mail address: eric.huang@hsc.utah.edu. (L.E. Huang).
[1] Current address: Children's Medical Center Research Institute at UT Southwestern.

http://dx.doi.org/10.1016/j.tranon.2020.100819

remaining wild-type allele) show drastic reduction of D-2HG [15]. Loss of *IDH1*^{R132H} heterozygosity occurs frequently in patient-derived xenografts and ex vivo spheroid cultures and is associated with glioma progression [3]. Compared with *IDH1*^{R132H}-heterozygous glioma cells, *IDH1*^{R132H}-hemizygous cells manifest robust anchorage-independent growth and aggressive tumor growth [15,16]. Furthermore, *IDH1*^{R132H}-hemizygous spheroid growth exhibits significant enrichment of the mesenchymal subtype gene set of glioblastoma and a transcriptomic profile resembling that of *IDH1*-wildtype glioma [16]. Although uncommon in glioma, loss of *IDH1*^{R132H} heterozygosity and copy number alterations at the *IDH1* locus are associated with glioma recurrence and progression [12,17]. Accordingly, we have proposed that loss of *IDH1*^{R132H} heterozygosity, but not necessarily *IDH1*^{R132H} itself, promotes glioma progression [3].

Previous studies showed that transgenic *IDH1*^{R132H} in contrast to wild-type *IDH1* induced nestin expression in immortalized human astrocytes, in correlation with general increases of DNA and histone methylation marks, a key piece of evidence for oncogenic transformation via blocking cell differentiation and adopting a stem-like phenotype [10,18]. Furthermore, treatment of TS603 glioma cells with the IDH1^{R132H} inhibitor AGI-5198 reduced the number of nestin-positive cells, thereby promoting differentiation [9]. These studies suggest the possibility of epigenetic modifications specific for *NES* (encoding nestin) upregulation in *IDH1*^{R132H}-heterozygous cells, even though the underlying mechanism remains unclear. Moreover, this finding is apparently at odds with the clinical observation that nestin is an adverse predictor whereas IDH1^{R132H} is a favorable predictor of survival in lower-grade glioma [19,20]. In this study, by taking advantage of the *IDH1*-wildtype-like phenotype of *IDH1*^{R132H} hemizygosity [16], we began by comparing nestin expression between *IDH1*^{R132H}-heterozygous and *IDH1*^{R132H}-hemizygous cells cultured in adherent and spheroid growth conditions to seek an in-depth understanding of neural stem-cell marker gene expression in relation to *IDH1*^{R132H} heterozygosity.

Materials and methods

Spheroid growth and treatment

BT142 mut/− (ATCC) was used to generate *IDH1*^{R132H}-heterozygous BT142 mut/IDH1 through a transgene that expresses YFP, P2A, and IDH1, whereas *IDH1*^{R132H}-hemizygous BT142 mut/YFP* was created similarly with an engineered stop codon at P2A [15]. *IDH1*^{R132H}-heterozygous (IMA mut/+) and *IDH1*-deleted *IDH1*^{R132H}-hemizygous (IMA mut/−) glioma cells were described previously [12]. Adherent and neural spheroid cultures were performed as described previously [15,21]. Briefly, adherent culture was maintained in a complete growth medium consisting of 45% stem cell medium, 45% DMEM, and 10% FBS, whereas spheroid culture used a neurobasal medium supplemented with B-27, 10 ng/mL bFGF, and 20 ng/mL EGF (Invitrogen).

To ensure adequate treatment with epigenetic agents, cells were first grown in adherent culture with 3 μM AGI-5198 (Sigma-Aldrich, St. Louis, MO, USA) in reference to DMSO or 1 mM octyl-(*R*)-2HG (Sigma-Aldrich) in reference to ethanol for 3 or 5 days. Likewise, 5 μM 5-aza-2′-deoxycytidine (DAC; TCI America, Portland, OR, USA), UNC0642, or UNC1999 (MedChemExpress, Monmouth, NJ, USA) was administered to adherent cells with medium replacement every other day for a total of 5 days in reference to vehicle control. Treated cells were subsequently seeded at a density of 5×10^4 per well in a 48-well plate for spheroid growth, with continued dosing every three days. Unless otherwise specified, spheroid growth was terminated 7 days after seeding for further analyses.

Gene expression

For reverse transcription–quantitative PCR, total RNA was extracted from *IDH1*^{R132H}-heterozygous and *IDH1*^{R132H}-hemizygous BT142 cells cultured under spheroid conditions in 3 biological replicates [16] and was converted to cDNA as previously described [15]. Quantitative PCR was performed in quadruplicate with LightScanner Master Mix (BioFire Diagnostics, Salt Lake City, UT, USA) using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The primer sets are listed in Supplementary Table 1. The annealing temperature was set at 63 °C for 45 cycles. Quantitation cycle (Cq) values were obtained through CFX Manager Software (Bio-Rad) and were normalized by the Cq values of three reference genes: *RPL30*, *YWHAZ*, and *UBC*.

Western blot analysis was performed essentially as described previously [15,22]. Spheroid cultures between 40 and 100 μm were collected with cell sifters. The same membranes were probed with various antibodies with dilutions as follows: 1:500 anti-Nestin (GenScript, Piscataway, NJ, USA), 1:500 anti-IDH1 (Biovision, Milpitas, CA, USA), and 1:500 anti-IDH1^{R132H} (EMD Millipore, Burlington, MA, USA).

Bisulfite sequencing

Bisulfite conversion was conducted with 500 ng of genomic DNA of BT142 cells using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA). The UCSC Genome Browser on Human DEC. 2013 (GRCH38. HG38) Assembly and MethPrimer [23] were used for primer design (Supplementary Table 2). After 30-cycle amplification, PCR products were purified using DNA Clean & Concentrator-5 Kit (Zymo Research) and ligated into pGEM-T vector for bacterial transformation and DNA sequencing.

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed essentially as described previously [24]. Three biological replicates of BT142 mut/YFP* and BT142 mut/IDH1 cells in spheroid culture were prepared with 2×10^6 cells per replicate. The antibodies used for immunoprecipitation were anti-H3K4me3 (EMD Millipore), anti-H3K27me3 (Cell Signaling, Danvers, MA, USA), and anti-H3 (Abcam, Cambridge, MA, USA). The levels of *NES* and *CD24* trimethylation at H3K4 and H3K27 were determined with quantitative PCR as described above and were normalized by the Cq values of matched samples immunoprecipitated with the anti-H3 antibody. The primer sets are listed in Supplementary Table 3. Annealing temperatures were set at 63 °C for 45 cycles.

Bioinformatic analysis

The genomic data sets of GSE16011 and The Cancer Genome Atlas (TCGA) Brain Lower Grade Glioma (TCGA-LGG) were acquired as described previously [25,26]. GSE16011 contains 136 cases of IDH-wild-type and 80 cases of IDH1-mutant gliomas of World Health Organization (WHO) grade II to grade IV, and TCGA-LGG contains 53 cases of IDH-wild-type and 233 cases of IDH-mutant gliomas of WHO grade II to grade III. Comparative analyses of gene expression and DNA methylation based on IDH status were performed as described previously [25]. Likewise, Pearson correlations between DNA methylation and gene expression were performed using Prism 8 (GraphPad, San Diego, CA, USA).

Survival

Kaplan–Meier overall survival analysis of the GSE16011 data set was performed using the R2: Genomic Analysis and Visualization Platform (http://r2.amc.nl) with the Kaplan scan. The *p* values were Bonferroni corrected. Log-rank (Mantel-Cox) tests were performed according to the *z*-scores of gene expression from the TCGA-LGG data set using Prism 8. Multivariate Cox regressions based on gene expression, sex, age, and tumor grades of the TCGA-LGG data set were performed using OncoLnc (http://www.oncolnc.org) with *p* values corrected by false-discovery rate (FDR).

Statistical analysis

Unpaired *t*-tests with Welch's correction were used for comparative analysis of genomic data sets. Quantitative PCR and bisulfite sequencing data were analyzed in unpaired *t*-tests with Welch's correction or one-way ANOVA using Prism 8. Two-tailed *p* values were used for statistical significance (ns, not significant; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; and $****p < 0.0001$).

Results

Pronounced NES upregulation in $IDH1^{R132H}$-hemizygous but not $IDH1^{R132H}$-heterozygous spheroids

Our recent study indicates an *IDH1*-wildtype-like phenotype in $IDH1^{R132H}$-hemizygous spheroids but not in those with restored $IDH1^{R132H}$ heterozygosity through an *IDH1*-wildtype transgene [15,16]. Furthermore, the *IDH1*-wildtype-like phenotype and the enrichment of glioblastoma mesenchymal subtype gene set were seen only in spheroid but not adherent culture [16]. Accordingly, we compared *NES* expression in relation to $IDH1^{R132H}$ heterozygosity in both culture conditions. In contrast to less than twofold upregulation in adherent $IDH1^{R132H}$-heterozygous BT142 cells, *NES* expression was upregulated by 16-fold in $IDH1^{R132H}$-hemizygous spheroid growth (Fig. 1A). Likewise, nearly 10-fold *NES* upregulation was seen in spheroid growth of $IDH1^{R132H}$-hemizygous IMA glioma cells (owing to genetic deletion of wild-type *IDH1*) [12,15] compared with $IDH1^{R132H}$-heterozygous spheroid growth (Fig. 1B). Of note, the striking increase of *NES* expression is specific to the spheroid culture of $IDH1^{R132H}$-hemizygous cells. Furthermore, nestin protein levels were readily detectable in the spheroid culture of $IDH1^{R132H}$-hemizygous, but not $IDH1^{R132H}$-heterozygous, cells (Fig. 1C), which is in agreement with nestin expression in $IDH1^{R132H}$-hemizygous, but not $IDH1^{R132H}$-heterozygous, orthotopic xenografts [16]. In keeping with this, there was a conspicuous loss or decrease of $IDH1^{R132H}$ protein levels in $IDH1^{R132H}$-heterozygous spheroid, but not adherent, growth accompanied by decreased total IDH1 levels. This finding is in agreement with the marked reduction of D-2HG levels in $IDH1^{R132H}$-heterozygous spheroids and the selection against $IDH1^{R132H}$ expression by an epigenetic mechanism during anchorage-independent growth [15,21]. Of note, glutamate was added to stimulate spheroid growth of $IDH1^{R132H}$-heterozygous cells [21] for the sake of yielding sufficient cell lysates; however, no obvious effects were observed on *NES* mRNA levels in the treated cells (Supplementary Fig. 1). Taken together, these results indicate that *NES* expression in spheroid growth is biologically more relevant than in adherent growth [3,16] and is consistent with stimulated $IDH1^{R132H}$-hemizygous spheroid growth but inhibited $IDH1^{R132H}$-heterozygous spheroid growth observed previously [15].

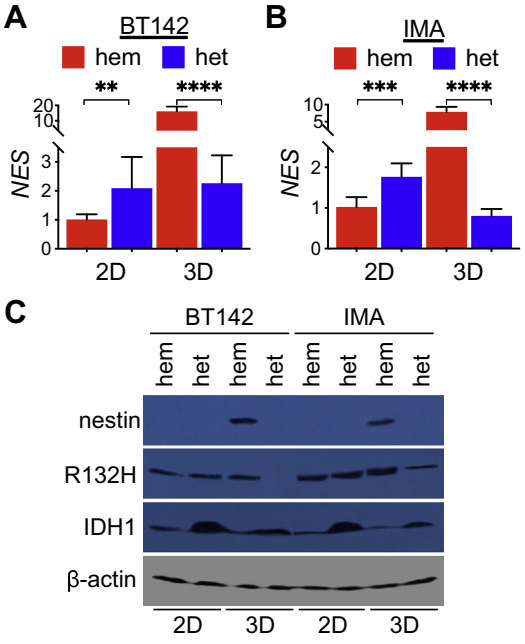

Fig. 1. Striking *NES* upregulation in $IDH1^{R132H}$-hemizygous spheroids. A and B, Quantitative PCR analysis showed marked increases of *NES* expression in $IDH1^{R132H}$-hemizygous (hem) BT142 (A, $n = 12$) and IMA (B, $n = 8$) spheroids (3D) in contrast to modest increases in $IDH1^{R132H}$-heterozygous (het) adherent cells (2D). C. Nestin protein abundance was detected in $IDH1^{R132H}$-hemizygous, but not $IDH1^{R132H}$-heterozygous, spheroids. Conspicuous loss or decrease of $IDH1^{R132H}$ protein levels in $IDH1^{R132H}$-heterozygous spheroids accompanied by decreased total IDH1 levels. Of note, sodium glutamate was used to facilitate spheroid growth of $IDH1^{R132H}$-heterozygous cells.

NES downregulation in IDH-mutant glioma

To test the notion that *NES* expression in spheroid growth is more relevant to glioma biology, we compared *NES* expression between IDH-mutant and IDH-wildtype gliomas using two independent genomic data sets: GSE16011 and TCGA-LGG. The mean *z*-scores of *NES* transcript in GSE16011 were 0.1790 and −0.3043 ($p = 0.0005$, 95% CI = −0.7507 to −0.2160) between IDH-wildtype and IDH-mutant samples (Fig. 2A). Furthermore, in agreement with the previous report that nestin is an adverse predictor of lower-grade gliomas [20], *NES* expression was negatively associated with overall survival (Supplementary Fig. 2A). Moreover, *NES* upregulation in IDH-wildtype glioma versus IDH-mutant glioma was confirmed in TCGA-LGG: 0.5967 versus −0.1799 ($p = 0.0002$, 95% CI = −0.7507 to −0.2160) (Fig. 2B). Together, these results not only indicate *NES* downregulation in IDH-mutant glioma, in agreement with better patient outcome, but also corroborate the biological relevance of spheroid culture to *NES* expression, which is repressed in $IDH1^{R132H}$-heterozygous cells but markedly upregulated upon loss of $IDH1^{R132H}$ heterozygosity.

CD24 upregulation specifically in IDH-mutant glioma

Our observation that *NES* expression is downregulated in IDH-mutant glioma compared with IDH-wildtype glioma is apparently at odds with the previous reports that nestin levels were much higher in immortalized

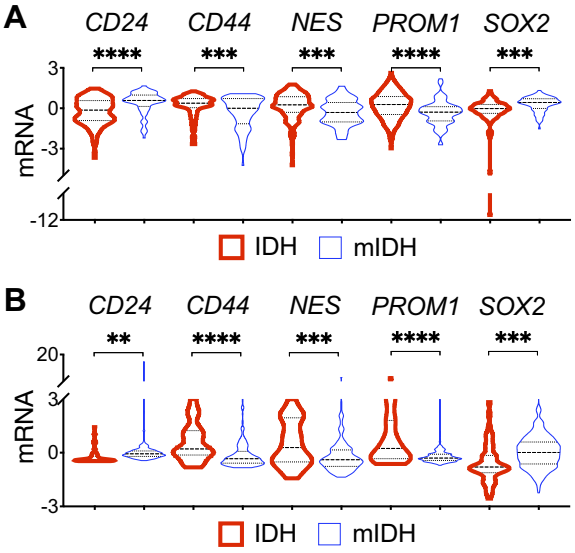

Fig. 2. Stem-cell marker gene expression in IDH-mutant glioma. A and B, Violin plots showing significant downregulation of *CD44*, *NES*, and *PROM1* but upregulation of *CD24* and *SOX2* in IDH-mutant glioma (mIDH) compared with IDH-wildtype (IDH) glioma from the GSE16011 (*A*) and TCGA-LGG (*B*) data sets.

human astrocytes transduced with *IDH1*^{R132H} in reference to wild-type *IDH1* [10,18]. To provide further evidence, we sought to examine the expression of additional glioma stem-cell marker genes, including *CD24*, *CD44*, *PROM1* (prominin 1, aka CD133), and *SOX2* (sex-determining region Y-box 2) in patient samples. Interestingly, we observed an extremely significant increase of mean *CD24* expression in IDH-mutant glioma of GSE16011; the mean *z*-scores of *CD24* transcript in IDH-wildtype and IDH-mutant samples were −0.2425 and 0.4122, respectively ($p < 0.0001$, 95% CI = 0.4011 to 0.9082), with a similar finding in TCGA-LGG: −0.2264 and 0.05148 ($p = 0.0021$, 95% CI = 0.1017 to 0.4541) (Fig. 2A, B). Furthermore, three- and fourfold increases of *CD24* expression were detected in *IDH1*^{R132H}-heterozygous BT142 and IMA spheroid growth compared with *IDH1*^{R132H}-hemizygous ones (Fig. 3A, B), which is similar to a previous finding of *CD24* upregulation in immortalized human astrocytes with long-term *IDH1*^{R132H} expression in reference to those without [27]. Moreover, we observed significant associations of *CD24* upregulation with better overall survival in both GSE16011 and TCGA-LGG (Fig. 3C, D). The result from multivariate Cox regression analysis confirmed the survival benefit of *CD24* expression with a significant Cox coefficient of −0.28 (Table 1), which is apparently in contrast to the implication of *CD24* in gliomagenesis [27].

Similar to *NES* downregulation in IDH-mutant glioma, both *CD44* and *PROM1* transcript levels were significantly lower in IDH-mutant glioma than in IDH-wildtype glioma (Fig. 2), and in *IDH1*^{R132H}-heterozygous BT142 and IMA spheroids than in *IDH1*^{R132H}-hemizygous ones (Fig. 3A, B). Both *CD44* and *PROM1* were negatively associated with overall survival, with a significant Cox coefficient of 0.40 and 0.26, respectively (Supplementary Fig. 2; Table 1). Oddly, *SOX2* transcript levels were significantly higher in IDH-mutant glioma than in IDH-wildtype glioma (Fig. 2) but significantly lower in *IDH1*^{R132H}-heterozygous BT142 and IMA spheroids than in *IDH1*^{R132H}-hemizygous ones (Fig. 3A, B). Furthermore, the association of *SOX2* expression with overall survival seemed ambiguous (Supplementary Fig. 2; Table 1). Nevertheless, these results support the notion that *CD24* is upregulated specifically among the neural stem-cell markers in IDH-mutant glioma whereas the downregulation of *CD44*, *NES*, and *PROM1* is in agreement with the better patient outcome.

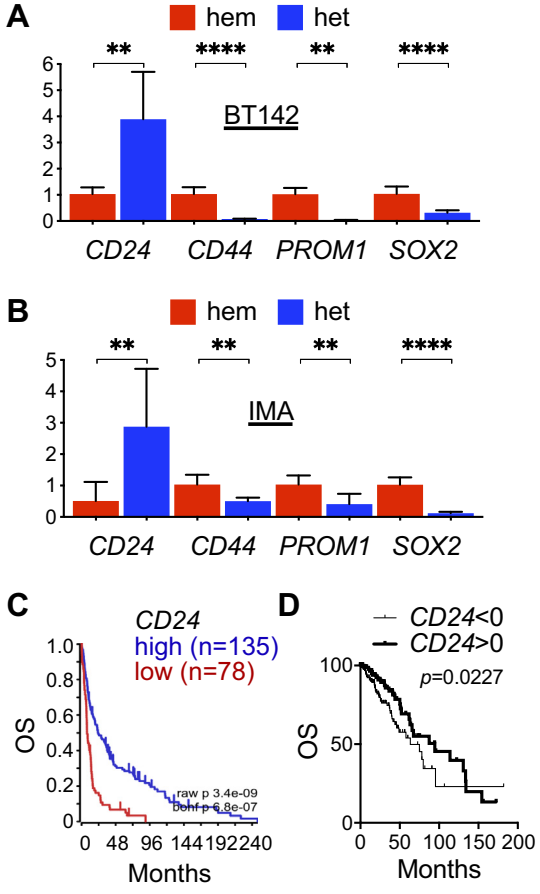

Fig. 3. *CD24* upregulation specifically in *IDH1*^{R132H}-heterozygous glioma cells. A and B. Quantitative PCR revealed significant upregulation of *CD24* but downregulation of other specified stem-cell marker genes in both *IDH1*^{R132H}-heterozygous BT142 (*A*) and IMA (*B*) spheroids compared with their *IDH1*^{R132H}-hemizygous controls ($n = 8$). C and D, *CD24* expression is associated with better overall survival (OS), as shown in Kaplan–Meier survival analysis of the GSE16011 data set with Bonferroni-corrected $p = 6.8e-07$ (C) and log-rank test of the TCGA-LGG data set (D).

Differential regulation of CD24 and NES expression by D-2HG

Previously, we showed a marked decrease of D-2HG in *IDH1*^{R132H}-hemizygous cells compared with *IDH1*^{R132H}-heterozygous cells [15]. Therefore, we sought to ascertain whether octyl-(*R*)-2HG—a membrane-permeant precursor form of D-2HG—could alter *CD24* and *NES* expression in *IDH1*^{R132H}-hemizygous cells. Indeed, such treatment led to an approximately fivefold increase of *CD24* expression but an eightfold decrease of *NES* expression in *IDH1*^{R132H}-hemizygous BT142 spheroids (Fig. 4A) along with nearly fourfold inhibition of spheroid growth (Supplementary Fig. 3A). In *IDH1*^{R132H}-heterozygous spheroids, however, such treatment had no effect on *CD24* expression but further decreased *NES* expression by tenfold (Fig. 4B) along with moderate inhibition of spheroid growth (Supplementary Fig. 3A). Conversely, treatment of *IDH1*^{R132H}-heterozygous spheroids with AGI-5198—a potent IDH1^{R132H} inhibitor [9]—resulted in 70% decrease in *CD24* expression, but a 14-fold increase of *NES* expression along with nearly threefold increase of spheroid growth (Supplementary Fig. 3B), in contrast to modest effects on *IDH1*^{R132H}-hemizygous spheroids (Fig. 4C, D). Consistently, AGI-5198 increased *CD44* expression in *IDH1*^{R132H}-heterozygous spheroids, whereas octyl-(*R*)-2HG inhibited *CD44* and *PROM1* expression in both types of spheroids. Similar results were obtained in IMA spheroids (Supplementary Figs. 3 and 4). Thus, our finding not only supports differential regulation of *CD24* and *NES* in *IDH1*^{R132H}-heterozygous cells but also suggests a distinct epigenetic mechanism underlying *CD24* upregulation.

Correlation of DNA methylation with neural stem-cell marker gene expression

IDH-mutant glioma is known to be associated with genome-wide DNA hypermethylation owing to D-2HG inhibition of the TET 5-methylcytosine hydroxylases, even though DNA hypomethylation also occurs to a lesser extent [28,29]. To provide evidence for an association of gene expression with epigenetic modification, we compared DNA methylation of neural stem-cell marker genes between IDH-mutant and IDH-wildtype gliomas using the TCGA-LGG data set. Whereas the mean values of DNA methylation for *CD44*, *NES*, *PROM1*, and *SOX2* in IDH-wildtype glioma were in a descending order (0.5980, 0.3635, 0.1381, and 0.09519), significant increases of DNA methylation (0.9023, 0.5389, 0.4503, and 0.04857) were evident in IDH-mutant glioma (Fig. 5A). Of note, *SOX2* remained essentially hypomethylated, with a minute difference in the mean values between the two glioma types. Furthermore, we observed significant inverse correlations between DNA methylation and gene expression in IDH-mutant glioma, albeit with *PROM1* showing relatively weak Pearson R^2 (Fig. 5B). In IDH-wildtype glioma, however, only *CD44* and *NES* showed extremely significant correlations. These results suggest that heterozygous *IDH1*^{R132H} induces the downregulation of neural stem-cell marker genes through epigenetic reprogramming.

Distinctive epigenetic modifications at the CD24 locus

To gain insight into epigenetic modification of *CD24*, which was unavailable from the TCGA-LGG data set, we performed bisulfite sequencing of *CD24* in reference to *NES* with genomic DNA extracted from *IDH1*^{R132H}-heterozygous and *IDH1*^{R132H}-hemizygous BT142 cells. Within the *CD24* promoter region examined, we observed an average of 28% CpG methylation in

Table 1
Cox regression analysis of listed genes from the TCGA-LGG data set.

Gene	Cox Coefficient[1]	FDR Corrected[2]
GLUD2	-0.32	5.73E-03
CD24	-0.28	4.89E-03
PDGFRA	-0.18	6.94E-02
ATM	-0.02	9.07E-01
SOX2	0.10	4.34E-01
NES	0.20	6.45E-02
PROM1	0.26	6.01E-03
CD44	0.40	2.66E-04
BCAT1	0.56	5.46E-07
IGFBP2	0.63	1.37E-07

[1]Listed in an ascending order.
[2]Insignificant FDR-corrected *p*-values indicated in gray.

IDH1^{R132H}-hemizygous cells versus 6% in *IDH1*^{R132H}-heterozygous cells—a more than fourfold decrease of DNA methylation (Fig. 6A, B). In contrast, the *NES* promoter region showed a greater than eightfold increase in DNA methylation in *IDH1*^{R132H}-heterozygous cells. This result suggests that the upregulation of *CD24* in IDH-mutant glioma is associated with a decrease of DNA methylation.

Moreover, we assessed specific changes in histone methylation at the gene locus of *CD24* in reference to *NES* using the transcriptional activation mark—trimethylation of histone 3 lysine 4 (H3K4me3)—and the repression mark—trimethylation of histone 3 lysine 27 (H3K27me3) [30]. Chromatin immunoprecipitation revealed that *IDH1*^{R132H}-heterozygous spheroid growth exhibited a 6-fold decrease of H3K27me3 at the *CD24* locus while a ~5.5-fold increase at the *NES* locus compared with *IDH1*^{R132H}-hemizygous spheroid growth (Fig. 6C). Interestingly, H3K4me3 was increased for both genes, suggesting the possibility of bivalent histone modifications [31]. Taken together, these results support the notion that heterozygous *IDH1*^{R132H} induces epigenetic modifications specific for *CD24* upregulation, whereas loss of *IDH1*^{R132H} heterozygosity alters epigenetic modifications to promote *IDH1*-wildtype-like gene expression.

Complex effects of epigenetic inhibitors on neural stem-cell marker gene expression

To test the functional relevance of epigenetic modifications to gene expression, we employed inhibitors of DNA methyltransferase and histone

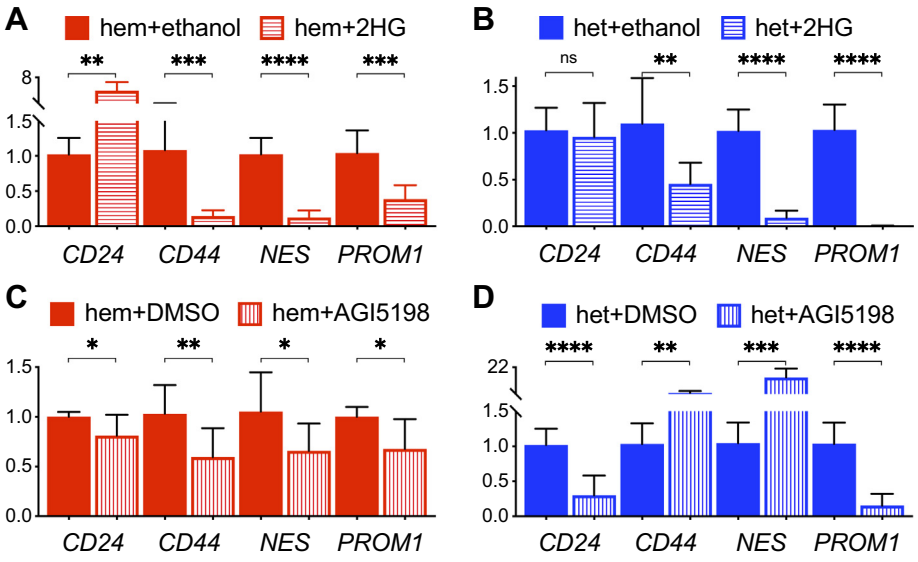

Fig. 4. Differential regulation of *CD24* and *NES* by D-2HG in BT142 spheroid growth. A and B, Octyl-(*R*)-2HG treatment stimulated *CD24* expression in *IDH1*^{R132H}-hemizygous spheroids (A, hem + 2HG) but inhibited *NES* expression in both *IDH1*^{R132H}-hemizygous and *IDH1*^{R132H}-heterozygous spheroids (B, het + 2HG) in reference to vehicle treatment (+ethanol). C and D, In contrast to modest effects in *IDH1*^{R132H}-hemizygous spheroids (C, hem + AGI5198), AGI-5198 treatment stimulated *NES* expression but inhibited *CD24* expression in *IDH1*^{R132H}-heterozygous spheroids (D, het + AGI5198) in reference to vehicle treatment (+DMSO). Gene expression was assayed with quantitative PCR (n = 8).

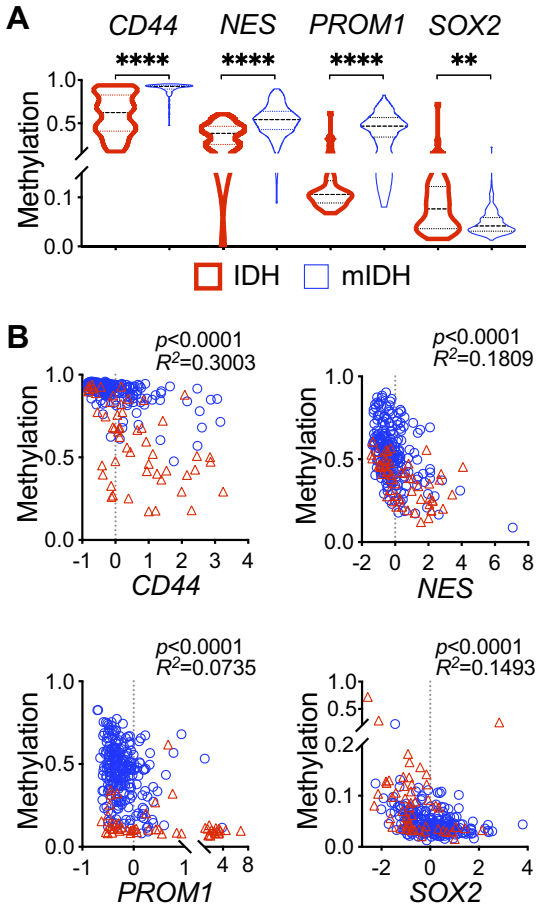

Fig. 5. Inverse correlations of DNA methylation with glioma stem-cell marker gene expression. A, Comparative analysis of the TCGA-LGG data set showing significantly higher median levels of DNA methylation in *CD44*, *NES*, and *PROM1* but lower in *SOX2* in IDH-mutant (mIDH) glioma compared with IDH-wildtype (IDH) glioma. B, Pearson correlations showing inverse correlations between DNA methylation and gene expression in IDH-mutant glioma (circles), as indicated by p and R^2 values, and also in IDH-wildtype glioma (triangles).

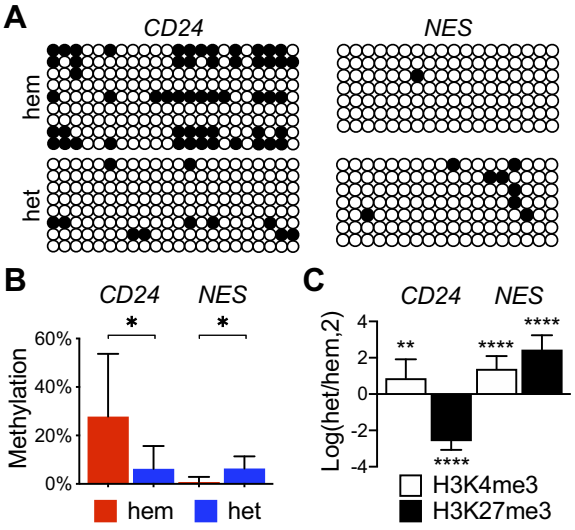

Fig. 6. Differential epigenetic modifications between *CD24* and *NES* in $IDH1^{R132H}$-heterozygous cells. A, Bisulfite sequencing results showing lower levels of CpG methylation (filled circles) in *CD24* but higher levels in *NES* in $IDH1^{R132H}$-heterozygous cells than in $IDH1^{R132H}$-hemizygous cells. B, Quantitative analysis of the bisulfite sequencing results. C, Chromatin immunoprecipitation of BT142 spheroids revealed an opposing trend of H3K27me3 ($n = 9$) between *CD24* and *NES*, presented in a log2 ratio of $IDH1^{R132H}$-heterozygous cells over $IDH1^{R132H}$-hemizygous cells.

demethylases in $IDH1^{R132H}$-heterozygous cells. Treatment with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine resulted in a general downregulation of glioma stem-cell marker genes in comparison with the vehicle controls (Fig. 7A). This finding apparently differs from increased tissue factor (*F3*) expression after 5-aza-2′-deoxycytidine treatment [32] but may support the previous finding that treatment with 5-azacytidine effectively results in glioma regression [33]. Treatment with the histone methyltransferases G9a/GLP inhibitor UNC0642, which has been shown to reverse histone H3K9 methylation and restore *Atm* expression [34], yielded a modest increase in *NES* expression but downregulation of the other genes (Fig. 7B). However, treatment with the histone methyltransferase EZH2/EZH1 inhibitor UNC1999, which removes H3K27me3, resulted in a reversal of gene expression—*CD44* and *NES* upregulation but *CD24* and *PROM1* downregulation (Fig. 7C). Although the results indicate differential mechanisms of epigenetic regulation in IDH-mutant glioma, the precise mechanism for individual genes requires further investigation.

Discussion

The transgenic $IDH1^{R132H}$ effect on nestin expression was a key piece of evidence supporting the notion that $IDH1^{R132H}$ induces oncogenic transformation through epigenetic changes that block neural differentiation and adopt a stem-like phenotype [9,10,18]. However, our analyses of patient data and DNA and histone modifications indicate *NES* downregulation in IDH-mutant gliomas and $IDH1^{R132H}$-heterozygous spheroid growth compared respectively with IDH-wildtype gliomas and $IDH1^{R132H}$-hemizygous spheroid growth. Furthermore, *NES* expression is well correlated with epigenetic modifications, supporting epigenetic inhibition of *NES* expression in IDH-mutant glioma. The *NES* downregulation is in agreement with glioma patient outcome, which is associated negatively with nestin but positively with $IDH1^{R132H}$ [6,19,20]. Moreover, the general downregulation of neural stem-cell marker genes in IDH-mutant glioma compared with IDH-wildtype glioma is consistent with the inhibition of anchorage-independent growth of $IDH1^{R132H}$-heterozygous cells [15,16] and the retardation of glioma growth [16,35,36]. The modest increase of *NES* expression in adherent $IDH1^{R132H}$-heterozygous cells compared with $IDH1^{R132H}$-hemizygous cells, however, might explain the previous finding [9,10,18] presumably obtained under similar growth conditions. In light of our recent studies that anchorage-independent growth is more relevant to $IDH1^{R132H}$ glioma biology [15,16], we conclude therefore that heterozygous $IDH1^{R132H}$ epigenetically suppresses *NES* expression.

Of note, despite *NES* downregulation in IDH-mutant glioma, our studies also suggest that nestin is nonessential to anchorage-independent growth and tumor growth. We provided evidence here that although extracellular glutamate stimulated $IDH1^{R132H}$-heterozygous cells for spheroid growth, no *NES* upregulation was detected at the transcript and protein levels. Likewise, in orthotopic transplantation models, nestin was expressed in tumors derived from $IDH1^{R132H}$-hemizygous, but not $IDH1^{R132H}$-heterozygous, glioma cells [16]. Thus, these findings not only are consistent with the term of stem-cell marker but, more importantly, support the notion of a tissue-specific role for glutamate in $IDH1^{R132H}$ glioma biology [3,16,21].

Interestingly, heterozygous $IDH1^{R132H}$ induced *CD24* upregulation in stark contrast to the general downregulation of glioma stem-cell marker genes. CD24—a glycosylphosphatidylinositol-anchored molecule—is a marker of neural cell lineage tumors [37] and has been implicated in $IDH1^{R132H}$ gliomagenesis [27]. Likewise, *CD24* expression is generally associated with cancer progression and poor survival including glioblastoma [38,39]. Furthermore, CD24 has been identified as a novel 'don't eat me' signal of cancer cells through the interaction with Siglec-10 expressed in tumor-associated macrophages [40]. Surprisingly, we observed an association of *CD24* expression with better survival in both single- and multi-

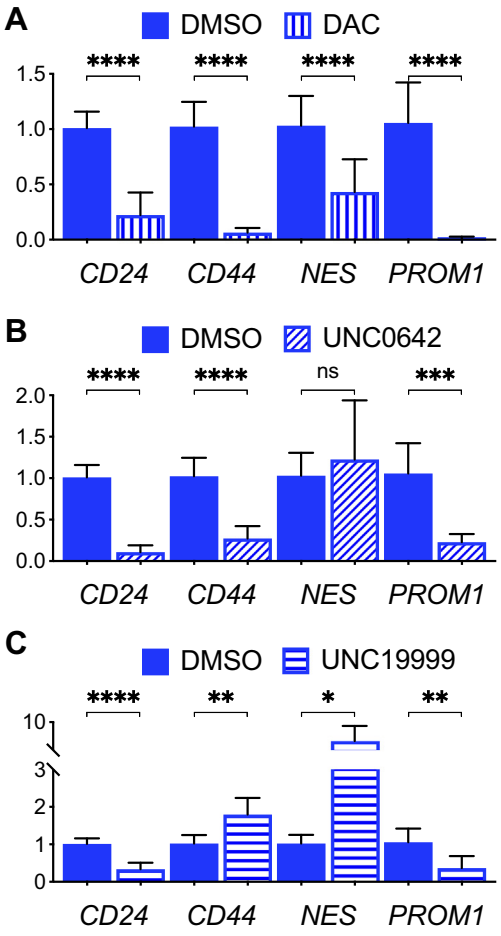

Fig. 7. Effects of epigenetic inhibitors on stem-cell marker gene expression in $IDH1^{R132H}$-heterozygous BT142 spheroids. Quantitative PCR analysis showed general inhibition of gene expression by 5-aza-2′-deoxycytidine (*DAC*, A) and UNC0642 except for *NES* (B), and stimulation of *CD44* and *NES* but inhibition of *CD24* and *PROM1* by UNC1999 (C) in reference to DMSO treatment. One-way ANOVA was performed by comparing with vehicle treatment.

variate analyses. Of note, intracellular *CD24* expression is required for the inactivation of remnant activity of mutant TP53, and high-level *CD24* expression is associated with *TP53* mutations in TCGA lower-grade glioma [41]. Moreover, *CD24* expresses differential isoforms according to the tissue of origin, differentiation status, and post-translational modifications [37]. Therefore, further studies are warranted to unravel the molecular intricacies between *TP53* mutations, *CD24* upregulation, and CD24 isoforms.

Mechanistically, we provided evidence that $IDH1^{R132H}$-heterozygous cells not only responded to AGI-5198 by decreasing *CD24* expression while increasing *NES* expression but also exhibited DNA hypomethylation at the *CD24* locus, consistent with the previous reporting of the existence of DNA hypomethylation despite genome-wide DNA hypermethylation in IDH-mutant glioma [28,29]. Conversely, octyl-(*R*)-2HG treatment specifically increased *CD24* expression in $IDH1^{R132H}$-hemizygous cells. Furthermore, the divergent trend of DNA methylation and gene expression between *CD24* and *NES* is in good agreement with the levels of transcriptional repressive H3K27me3—significantly lower in *CD24* but much higher in *NES*. Further investigations are required, however, to determine whether the H3K27me3 level is key to the differential expression of *CD24* and *NES* because the H3K27me3 inhibitor UNC1999 increased the expression of *NES* and *CD44* but, unexpectedly, decreased the expression of *CD24* in $IDH1^{R132H}$-heterozygous cells. Moreover, previous studies showed that the accumulation of H3K9me3—another histone-repressive mark—was particularly noticeable in IDH-mutant cells [18] and the use of H3K9me3 inhibitor UNC0642 reduced H3K9 methylation and increased *Atm* expression [34]. The consequence of UNC0642 treatment in this study, however, was gene downregulation in general, suggesting the complexity of epigenetic regulation in IDH-mutant cells. An integrated genomic approach is expected to reveal how differential gene expression in response to these treatments correlates with epigenetic changes of these neural stem cell marker genes. Nevertheless, our results support the notion that *CD24* is upregulated specifically in IDH-mutant glioma in association with epigenetic modifications.

A relatively small number of genes are known to be markedly upregulated and biologically relevant to $IDH1^{R132H}$ glioma. *GLUD2* (glutamate dehydrogenase 2) is upregulated in IDH-mutant glioma to alleviate *IDH1* mutation-induced metabolic stress and promote glioma growth [42,43]. Interestingly, *GLUD2* upregulation also has positive correlation with patient survival (Table 1; data not shown). *PDGFRA* is another example of aberrant upregulation via DNA hypermethylation that compromises the binding of methylation-sensitive insulator protein CTCF for gene activation [44], but whether *PDGFRA* upregulation promotes IDH-mutant gliomagenesis requires further investigation, especially considering its association with better overall survival [25] and has a negative Cox coefficient in multivariate analysis (Table 1). Moreover, *ATM* (ataxia telangiectasia mutated) upregulation was reported in a mouse model of $IDH1^{R132H}$ glioma with *Trp53* and *Atrx* mutations [45], but *Atm* downregulation was shown in a myeloid lineage-specific conditional *Idh1*-mutated mouse model [34]. Nonetheless, the prognostic value of *ATM* expression in glioma patients seems less clear (Table 1; data not shown).

In sum, we presented evidence for the general downregulation of neural stem-cell marker genes including nestin in IDH-mutant glioma but markedly upregulated upon loss of $IDH1^{R132H}$ heterozygosity. However, *CD24* is specifically upregulated in IDH-mutant glioma in association with reduced DNA methylation and histone-repressive mark. Further studies are warranted to determine the underlying mechanism and biological role of *CD24* expression in IDH-mutant glioma.

Declaration of competing interest

None.

Acknowledgments

The authors thank Luming Zhou and Carl T. Wittwer for the assistance in quantitative PCR analysis and Kristin Kraus for editorial assistance.

Funding sources

This work was supported in part by NIH R21NS108065 (LEH), NIH R21HG009181 (MBC), and NIH R01CA188520 (SB).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2020.100819.

References

[1] Q.T. Ostrom, L. Bauchet, F.G. Davis, et al., The epidemiology of glioma in adults: a "state of the science" review, Neuro-Oncol. 16 (2014) 896–913 https://doi.org/10.1093/neuonc/nou087.
[2] P.Y. Wen, S. Kesari, Malignant gliomas in adults, N. Engl. J. Med. 359 (2008) 492–507 https://doi.org/10.1056/nejmra0708126.
[3] L.E. Huang, Friend or foe—IDH1 mutations in glioma 10 years on, Carcinogenesis 40 (2019) 1299–1307 https://doi.org/10.1093/carcin/bgz134.
[4] L. Dang, D.W. White, S. Gross, et al., Cancer-associated IDH1 mutations produce 2-hydroxyglutarate, Nature 462 (2009) 739–744 https://doi.org/10.1038/nature08617.
[5] P.S. Ward, J. Patel, D.R. Wise, et al., The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting α-ketoglutarate to 2-hydroxyglutarate, Cancer Cell 17 (2010) 225–234 https://doi.org/10.1016/j.ccr.2010.01.020.
[6] H. Yan, D.W. Parsons, G. Jin, et al., IDH1 and IDH2 mutations in gliomas, N. Engl. J. Med. 360 (2009) 765–773 https://doi.org/10.1056/nejmoa0808710.

[7] X. Tang, X. Fu, Y. Liu, et al., Blockade of glutathione metabolism in IDH1-mutated glioma, Mol. Cancer Ther. 19 (2019) 221–230 https://doi.org/10.1158/1535-7163.mct-19-0103.
[8] W. Xu, H. Yang, Y. Liu, et al., Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of α-ketoglutarate-dependent dioxygenases, Cancer Cell 19 (2011) 17–30 https://doi.org/10.1016/j.ccr.2010.12.014.
[9] D. Rohle, J. Popovici-Muller, N. Palaskas, et al., An inhibitor of mutant IDH1 delays growth and promotes differentiation of glioma cells, Science 340 (2013) 626–630 https://doi.org/10.1126/science.1236062.
[10] S. Turcan, D. Rohle, A. Goenka, et al., IDH1 mutation is sufficient to establish the glioma hypermethylator phenotype, Nature 483 (2012) 479–483 https://doi.org/10.1038/nature10866.
[11] F. Wang, J. Travins, B. DeLaBarre, et al., Targeted inhibition of mutant IDH2 in leukemia cells induces cellular differentiation, Science 340 (2013) 622–626 https://doi.org/10.1126/science.1234769.
[12] G. Jin, Z.J. Reitman, C.G. Duncan, et al., Disruption of wild-type IDH1 suppresses D-2-hydroxyglutarate production in IDH1-mutated gliomas, Cancer Research 73 (2013) 496–501 https://doi.org/10.1158/0008-5472.can-12-2852.
[13] P.S. Ward, C. Lu, J.R. Cross, et al., The potential for isocitrate dehydrogenase mutations to produce 2-hydroxyglutarate depends on allele specificity and subcellular compartmentalization, J. Biol. Chem. 288 (2013) 3804–3815 https://doi.org/10.1074/jbc.m112.435495.
[14] S. Han, Y. Liu, S.J. Cai, et al., IDH mutation in glioma: molecular mechanisms and potential therapeutic targets, Brit J Cancer 122 (2020) 1580–1589 https://doi.org/10.1038/s41416-020-0814-x.
[15] P.D.B. Tiburcio, B. Xiao, S. Berg, et al., Functional requirement of a wild-type allele for mutant IDH1 to suppress anchorage-independent growth through redox homeostasis, Acta Neuropathol. 135 (2018) 285–298 https://doi.org/10.1007/s00401-017-1800-0.
[16] P.D.B. Tiburcio, D.L. Gillespie, R.L. Jensen, L.E. Huang, Extracellular glutamate and IDH1R132H inhibitor promote glioma growth by boosting redox potential, J. Neuro-Oncol. 146 (2020) 427–437 https://doi.org/10.1007/s11060-019-03359-w.
[17] T. Mazor, C. Chesnelong, A. Pankov, et al., Clonal expansion and epigenetic reprogramming following deletion or amplification of mutant IDH1, Proc. Natl. Acad. Sci. 114 (2017) 10743–10748 https://doi.org/10.1073/pnas.1708914114.
[18] C. Lu, P.S. Ward, G.S. Kapoor, et al., IDH mutation impairs histone demethylation and results in a block to cell differentiation, Nature 483 (2012) 474–478 https://doi.org/10.1038/nature10860.
[19] M. Sanson, Y. Marie, S. Paris, et al., Isocitrate dehydrogenase 1 codon 132 mutation is an important prognostic biomarker in gliomas, J. Clin. Oncol. 27 (2009) 4150–4154 https://doi.org/10.1200/jco.2009.21.9832.
[20] K.J. Hatanpaa, T. Hu, V. Vemireddy, et al., High expression of the stem cell marker nestin is an adverse prognostic factor in WHO grade II-III astrocytomas and oligoastrocytomas, J. Neuro-oncol. 117 (2014) 183–189 https://doi.org/10.1007/s11060-014-1376-7.
[21] P.D.B. Tiburcio, B. Xiao, Y. Chai, et al., IDH1R132H is intrinsically tumor-suppressive but functionally attenuated by the glutamate-rich cerebral environment, Oncotarget 9 (2018) 35100–35,113 https://doi.org/10.18632/oncotarget.26203.
[22] H. Choi, D.L. Gillespie, S. Berg, et al., Intermittent induction of HIF-1α produces lasting effects on malignant progression independent of its continued expression, PLOS One 10 (2015), e0125125. https://doi.org/10.1371/journal.pone.0125125.
[23] L.-C. Li, R. Dahiya, MethPrimer: designing primers for methylation PCRs, Bioinformatics 18 (2002) 1427–1431 https://doi.org/10.1093/bioinformatics/18.11.1427.
[24] S. Bhaskara, S.K. Knutson, G. Jiang, et al., Hdac3 is essential for the maintenance of chromatin structure and genome stability, Cancer Cell 18 (2010) 436–447 https://doi.org/10.1016/j.ccr.2010.10.022.
[25] L.E. Huang, A.L. Cohen, H. Colman, et al., IGFBP2 expression predicts IDH-mutant glioma patient survival, Oncotarget 8 (2016) 191–202 https://doi.org/10.18632/oncotarget.13329.
[26] M. Karsy, J. Guan, L.E. Huang, Prognostic role of mitochondrial pyruvate carrier in isocitrate dehydrogenase-mutant glioma, J. Neurosurg. 130 (2018) 56–66 https://doi.org/10.3171/2017.9.jns172036.
[27] S. Turcan, V. Makarov, J. Taranda, et al., Mutant-IDH1-dependent chromatin state reprogramming, reversibility, and persistence, Nat. Genet. 50 (2018) 62–72 https://doi.org/10.1038/s41588-017-0001-z.
[28] C.G. Duncan, B.G. Barwick, G. Jin, et al., A heterozygous IDH1R132H/WT mutation induces genome-wide alterations in DNA methylation, Genome Res. 22 (2012) 2339–2355 https://doi.org/10.1101/gr.132738.111.
[29] S. Wei, J. Wang, O. Oyinlade, et al., Heterozygous IDH1R132H/WT created by "single base editing" inhibits human astroglial cell growth by downregulating YAP, Oncogene 37 (2018) 5160–5174 https://doi.org/10.1038/s41388-018-0334-9.
[30] Y. Shi, Histone lysine demethylases: emerging roles in development, physiology and disease, Nat. Rev. Genet. 8 (2007) 829–833 https://doi.org/10.1038/nrg2218.
[31] N.L. Vastenhouw, A.F. Schier, Bivalent histone modifications in early embryogenesis, Curr. Opin. Cell Biol. 24 (2012) 374–386 https://doi.org/10.1016/j.ceb.2012.03.009.
[32] D. Unruh, S. Mirkov, B. Wray, et al., Methylation-dependent tissue factor suppression contributes to the reduced malignancy of IDH1-mutant gliomas, Clin. Cancer Res. 25 (2018) 747–759 https://doi.org/10.1158/1078-0432.ccr-18-1222.
[33] A. Borodovsky, V. Salmasi, S. Turcan, et al., 5-azacytidine reduces methylation, promotes differentiation and induces tumor regression in a patient-derived IDH1 mutant glioma xenograft, Oncotarget 4 (2013) 1737–1747 https://doi.org/10.18632/oncotarget.1408.
[34] S. Inoue, W.Y. Li, A. Tseng, et al., Mutant IDH1 downregulates ATM and alters DNA repair and sensitivity to DNA damage independent of TET2, Cancer Cell 30 (2016) 337–348 https://doi.org/10.1016/j.ccell.2016.05.018.
[35] J. Anido, A. Sáez-Borderías, A. Gonzàlez-Juncà, et al., TGF-β receptor inhibitors target the CD44high/Id1high glioma-initiating cell population in human glioblastoma, Cancer Cell 18 (2010) 655–668 https://doi.org/10.1016/j.ccr.2010.10.023.
[36] S.K. Singh, C. Hawkins, I.D. Clarke, et al., Identification of human brain tumour initiating cells, Nature 432 (2004) 396–401 https://doi.org/10.1038/nature03128.
[37] C. Poncet, V. Frances, R. Gristina, et al., CD24, a glycosylphosphatidylinositol-anchored molecule, is transiently expressed during the development of human central nervous system and is a marker of human neural cell lineage tumors, Acta Neuropathol. 91 (1996) 400–408 https://doi.org/10.1007/s004010050442.
[38] T. Fukushima, T. Tezuka, T. Shimomura, et al., Silencing of insulin-like growth factor-binding protein-2 in human glioblastoma cells reduces both invasiveness and expression of progression-associated gene CD24, J. Biol. Chem. 282 (2007) 18634–18,644 https://doi.org/10.1074/jbc.m609567200.
[39] D.T. Gilliam, V. Menon, N.P. Bretz, J. Pruszak, The CD24 surface antigen in neural development and disease, Neurobiol. Dis. 99 (2017) 133–144 https://doi.org/10.1016/j.nbd.2016.12.011.
[40] A.A. Barkal, R.E. Brewer, M. Markovic, et al., CD24 signalling through macrophage Siglec-10 is a target for cancer immunotherapy, Nature 572 (2019) 392–396 https://doi.org/10.1038/s41586-019-1456-0.
[41] L. Wang, R. Liu, P. Ye, et al., Intracellular CD24 disrupts the ARF–NPM interaction and enables mutational and viral oncogene-mediated p53 inactivation, Nat. Commun. 6 (2015) 5909 https://doi.org/10.1038/ncomms6909.
[42] R. Chen, M.C. Nishimura, S. Kharbanda, et al., Hominoid-specific enzyme GLUD2 promotes growth of IDH1R132H glioma, P Natl Acad Sci Usa 111 (2014) 14217–14222 https://doi.org/10.1073/pnas.1409653111.
[43] M.S. Waitkus, C.J. Pirozzi, C.J. Moure, et al., Adaptive evolution of the GDH2 allosteric domain promotes gliomagenesis by resolving IDH1R132H induced metabolic liabilities, Cancer Res. 78 (2018) 36–50 https://doi.org/10.1158/0008-5472.can-17-1352.
[44] W.A. Flavahan, Y. Drier, B.B. Liau, et al., Insulator dysfunction and oncogene activation in IDH mutant gliomas, Nature 529 (2015) 110–114 https://doi.org/10.1038/nature16490.
[45] F.J. Núñez, F.M. Mendez, P. Kadiyala, et al., IDH1-R132H acts as a tumor suppressor in glioma via epigenetic up-regulation of the DNA damage response, Sci. Transl. Med. 11 (2019) eaaq1427 https://doi.org/10.1126/scitranslmed.aaq1427.