

Priority Report

Disruption of Wild-Type IDH1 Suppresses D-2-Hydroxyglutarate Production in IDH1-Mutated Gliomas

Genglin Jin¹, Zachary J. Reitman¹, Christopher G. Duncan¹, Ivan Spasojevic², David M. Gooden³, B. Ahmed Rasheed¹, Rui Yang¹, Giselle Y. Lopez¹, Yiping He¹, Roger E. McLendon¹, Darell D. Bigner¹, and Hai Yan¹

Abstract

Point mutations at Arg132 of the cytoplasmic NADP⁺-dependent isocitrate dehydrogenase 1 (IDH1) occur frequently in gliomas and result in a gain of function to produce the "oncometabolite" D-2-hydroxyglutarate (D-2HG). The mutated *IDH1* allele is usually associated with a wild-type *IDH1* allele (heterozygous) in cancer. Here, we identify 2 gliomas that underwent loss of the wild-type *IDH1* allele but retained the mutant *IDH1* allele following tumor progression from World Health Organization (WHO) grade III anaplastic astrocytomas to WHO grade IV glioblastomas. Intratumoral D-2HG was 14-fold lower in the glioblastomas lacking wild-type *IDH1* than in glioblastomas with heterozygous *IDH1* mutations. To characterize the contribution of wild-type *IDH1* to cancer cell D-2HG production, we established an *IDH1*-mutated astrocytoma (IMA) cell line from a WHO grade III anaplastic astrocytoma. Disruption of the wild-type *IDH1* allele in IMA cells by gene targeting resulted in an 87-fold decrease in cellular D-2HG levels, showing that both wild-type and mutant *IDH1* alleles are required for D-2HG production in glioma cells. Expression of wild-type IDH1 was also critical for mutant IDH1-associated D-2HG production in the colorectal cancer cell line HCT116. These insights may aid in the development of therapeutic strategies to target *IDH1*-mutated cancers. *Cancer Res*; 73(2); 496–501. ©2012 AACR.

Introduction

Recent exomic sequencing identified frequent mutations in *IDH1*, or in its homolog *IDH2*, in gliomas, and other cancers (1–3). Almost all *IDH1* mutations result in an amino acid substitution at R132. These mutations impair the physiologic function of IDH1 to convert isocitrate to α -ketoglutarate (α KG) and confer a gain of function to convert α KG to D-2-hydroxyglutarate (D-2HG), which accumulates to extremely high levels in tumors with *IDH1* mutations (~100-fold increase; refs. 2, 4, 5). D-2HG inhibits α KG-dependent dioxygenases, including Jumonji C domain-containing histone demethylases and Tet 5-methylcytosine (5 mC) hydroxylases, resulting in epigenetic alterations and perturbed cellular differentiation that may contribute to tumorigenesis (6–9). These observa-

tions suggest that patients with glioma may benefit from therapeutic inhibition of D-2HG production (4).

As IDH1 functions as a homodimer, a critical question is whether IDH1 mutants exert their biologic function as mutant:mutant homodimers and/or as mutant:wild-type heterodimers. We showed using co-immunoprecipitation that wild-type IDH1 can bind to mutant IDH1 in glioma cells (5). Also, the wild-type:mutant IDH1 heterodimer produces D-2HG at a faster rate than the mutant:mutant homodimer under specific *in vitro* reaction conditions (10, 11). While wild-type IDH1 can produce low levels of D-2HG at a slow rate on its own (11, 12), it is unknown whether wild-type IDH1 is required for mutant IDH1 to elicit the extremely high D-2HG found in IDH1-mutated tumor cells.

Here, we report that loss of wild-type IDH1 was associated with a dramatic decrease in D-2HG in 2 *IDH1*-mutated astrocytomas. We show in a novel IDH1-mutated astrocytoma cell line and a colon cancer cell line that expression of wild-type IDH1 is required to produce high levels of D-2HG. These findings reveal that wild-type IDH1 contributes to D-2HG production in the glioma cellular environment.

Materials and Methods

Ethics statement and patient samples

Tumors were obtained from the Brain Tumor Biorepository at Duke with written informed consent from patients and Institutional Review Board approval and analyzed previously for *IDH* mutation status (2). Short tandem repeat genotyping was conducted with the AmpFISTR Identifier Kit (Applied Biosystems).

Authors' Affiliations: ¹The Preston Robert Tisch Brain Tumor Center, The Pediatric Brain Tumor Foundation Institute, and The Department of Pathology, ²The Clinical Pharmacology Laboratory, Duke Cancer Institute and Department of Medicine/Oncology, and ³The Small Molecule Synthesis Facility, Department of Chemistry, Duke University Medical Center, Durham, North Carolina

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G. Jin and Z. J. Reitman contributed equally to this work.

Corresponding Author: Hai Yan, Department of Pathology, Duke University Medical Center, DUMC-3156, 199B-MSRB, Research Drive, Durham, NC 27710. Phone: 919-668-7850; Fax: 919-684-8756; E-mail: hai.yan@duke.edu

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Cell lines

IDH1-mutated astrocytoma (IMA) cell line was derived from a 26-year-old male anaplastic astrocytoma WHO grade III patient treated at Duke in 2009. Tissue was dissociated by 100 μ g/mL liberase and cultured in stem cell medium [knockout minimum essential medium supplemented with 20 ng/mL EGF, 50 ng/mL basic fibroblast growth factor (bFGF), B-27 without Vitamin A supplement (1:50, Life Technologies), GlutaMAX (1:100, Life Technologies), nonessential amino acids solution (1:100, Life Technologies), 10 ng/mL leukocyte inhibitory factor, 2 μ g/mL heparin] as floating spheres. After 2.5 months, cells were transferred to 45% stem cell medium, 45% DMEM, 10% FBS and grew as monolayers. After 9 months, cells were monocloned and *IDH1* gDNA and cDNA was sequenced.

IMA cells were tested and authenticated by Sanger sequencing of *IDH1*, *TP53*, and *ATRX* as well as AmpFISTR genotyping as recently as May, 2012. Differentiation status was evaluated with mouse anti-GFAP (1:100, 556328 BD Biosciences), mouse anti-Tuj1 (1:200, MMS-435P Covance), rabbit anti-Sox2 (1:150, sc-20978 Santa Cruz), and rabbit anti-Nestin (1:200, AB5603 Chemicon) with Oregon Green 488 anti-rabbit (1:200, 11038 Life Technologies) or Alexa Fluor 350 anti-mouse (1:200, A10035 Life Technologies) secondary antibodies by immunofluorescence (Nikon TE2000-E microscope). Cell growth was compared by assessing viability using the MTT assay (13, 14). Genomic DNA content was assessed by qPCR as described previously (14). Apoptosis was evaluated with ApoDetect Annexin V-FITC Kit (Life Technologies) by immunofluorescence.

Knockout and overexpression

Knockout generation was conducted as described (15). *IDH1*^{WT} or *IDH1*^{R132H} was ectopically expressed using lentivirus constructs described previously (16) after 1-month selection with 0.5 μ g/mL blasticidin and expression was assessed by Western blotting. Anti-IDHC (Santa Cruz, N-20) and anti-*IDH1*^{R132H} (Dianova) were used with anti-GAPDH (Santa Cruz, FL-335) followed by horseradish peroxidase (HRP)-conjugated secondary antibody and chemiluminescence detection.

Metabolite quantification

Octyl- α KG synthesis is detailed in Supplementary Fig. S1 (7). D-2HG was quantified by liquid chromatography/tandem mass spectrometry (LC/MS-MS; ref. 5) and α KG was quantified using the α KG Assay Kit (BioVision, K677-100).

Results

Loss of *IDH1*^{WT} is associated with lowered tumoral D-2HG

Among 494 central nervous system tumors used to survey the frequency of *IDH1* mutations, we identified 2 WHO grade IV secondary glioblastomas with *IDH1*^{R132H} mutations in which a wild-type *IDH1* allele was undetectable by Sanger sequencing (2). Both tumors had progressed from WHO grade III anaplastic astrocytomas in which wild-type and mutant *IDH1* alleles (heterozygous) were present (2). Short tandem repeat marker analysis revealed that loss of hetero-

zygosity of D2S1338 (located at 2q35) occurred after progression of those tumors (Fig. 1A). These results indicate that the chromosomal region containing wild-type *IDH1* (located at 2q34) was deleted, but mutant *IDH1* was retained (*IDH1*^{R132H/-}) during progression. The *IDH1*^{R132H/-} tumors had 8-fold lower mean intratumoral D-2HG than in the tumors from which they progressed (Fig. 1A).

We next compared the D-2HG levels in the *IDH1*^{R132H/-} tumors to the D-2HG levels in other WHO grade IV glioblastomas for which tissue was available (Fig. 1B and Supplementary Table S1). As expected on the basis of previous findings (4, 5), the *IDH1*^{R132H/WT} glioblastomas had high D-2HG levels ($n = 7$, mean 103 mg/g protein) than the *IDH1*^{WT/WT} glioblastomas ($n = 8$, mean 0.241 mg/g protein, $P < 0.005$; t test). The *IDH1*^{R132H/-} glioblastomas had 14-fold lower mean D-2HG than *IDH1*^{R132H/WT} glioblastomas. Similar results were found when comparing the *IDH1*^{R132H/-} glioblastomas to grade II-III gliomas that we analyzed previously ($n = 11$; ref. 5), which are also displayed in Fig. 1B. The finding that *IDH1*^{R132H/-} gliomas had lower D-2HG than *IDH1*^{R132H/WT} gliomas led us to hypothesize that efficient D-2HG production in glioma cells may require both wild-type *IDH1* and mutant *IDH1* alleles.

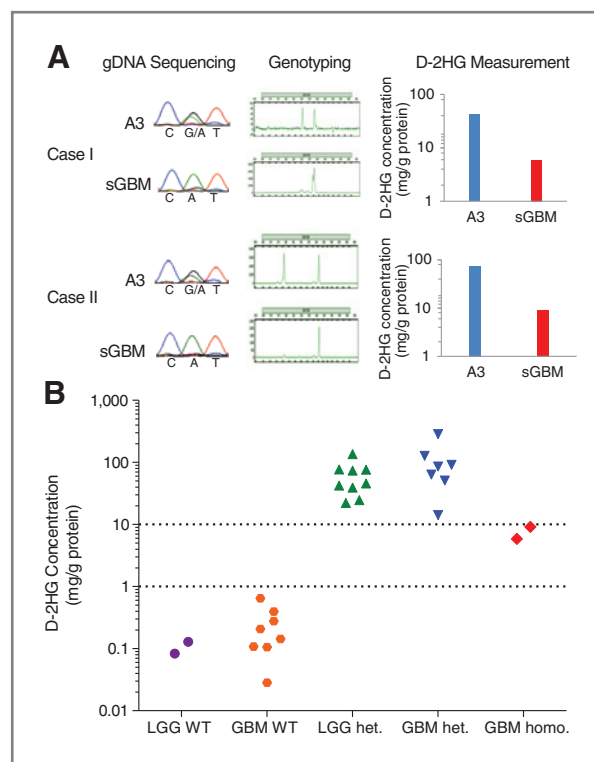


Figure 1. *IDH1*^{R132H/-} gliomas have lower D-2HG than *IDH1*^{R132H/WT} gliomas. A, two anaplastic astrocytomas WHO grade III (A3) that progressed to glioblastomas WHO grade IV (sGBM) show loss of the wild-type *IDH1* allele and decreased levels of D-2HG. Mutant *IDH1*^{R132H} is CAT (His); wild-type *IDH1* is CGT (Arg). B, D-2HG levels in human gliomas with wild-type *IDH1* (WT/WT), heterozygous *IDH1* mutation (R132H/WT), or *IDH1* mutation with loss of wild-type *IDH1* (R132H/-). LGG, lower grade glioma WHO grade II or III.

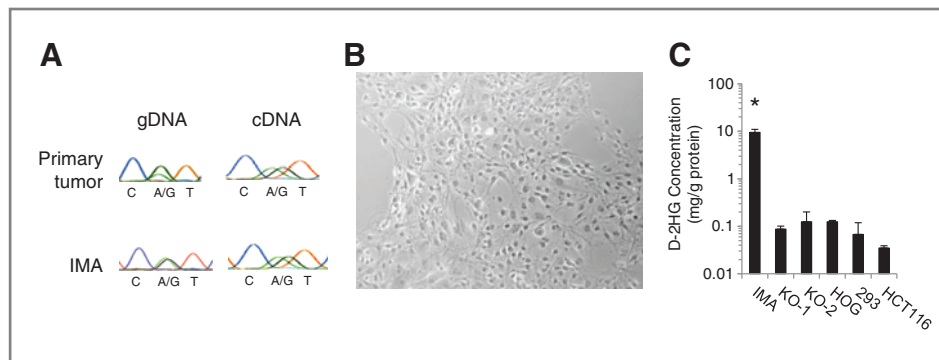


Figure 2. Establishment of an IMA. A, representative sequencing chromatograms for *IDH1* codon 132 in gDNA and cDNA. Both the primary tumor and IMA are heterozygous for wild-type (CGT) and mutant (CAT) alleles coding for an arginine (R) to histidine (H) change at amino acid residue 132. B, bright-field image of adherent IMA cells. C, D-2HG level in lysates of IMA (*IDH1*^{R132H/WT}), 2 *IDH1* wild-type allele knocked out subclones KO-1 and KO-2 (*IDH1*^{R132H/-}), and HCT116, HOG, and 293 cells, which do not contain *IDH1* mutations. *, D-2HG was significantly higher in IMA for pairwise comparisons with each other cell line ($P < 0.0001$ for each, Student *t* test). Mean \pm SD are shown from lysates collected in triplicate.

Establishment of IMA

To examine whether *IDH1*^{WT} was needed along with *IDH1*^{R132H} to produce D-2HG in glioma cells, we derived IMA, an anaplastic astrocytoma WHO grade III cell line containing a

native, heterozygous *IDH1* mutation (*IDH1*^{R132H/WT}; Fig. 2A). IMA also contained stable mutations in *TP53* (p.G245V) and in *ATRX* (p.R781X). No copy number alterations were detected in genomic loci that are frequently altered in primary

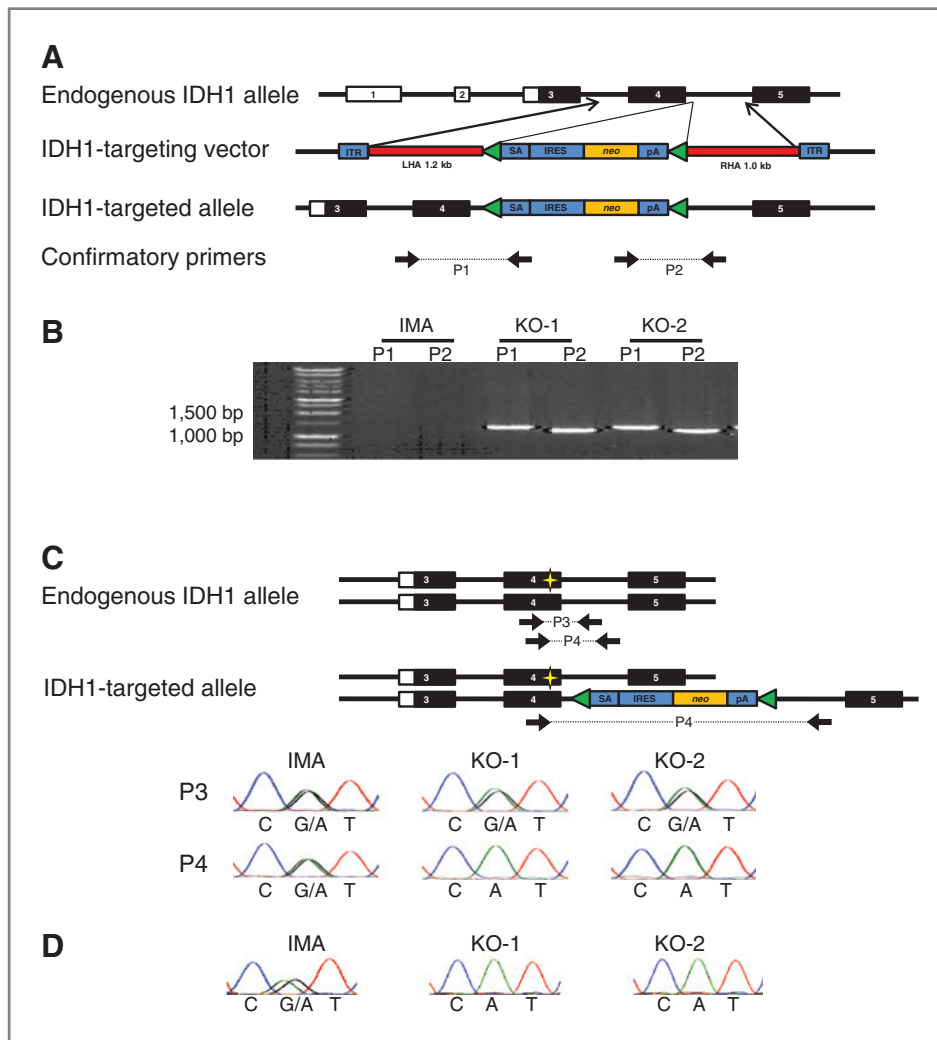


Figure 3. Targeted knockout of wild-type *IDH1* in IMA. A, targeting vector used to disrupt expression of one allele of *IDH1*. The targeting vector contains a splice acceptor (SA), internal ribosomal entry sequence (IRES), neomycin selectable marker (*neo*), and a polyadenylation site (pA), all flanked by right and left homology arms (LHA and RHA, respectively) and inverted terminal repeats (ITR). B, diagnostic PCR confirms homologous integration of the targeting vector into the *IDH1* genomic locus for clones KO-1 and KO-2 but not for the parental IMA cells. Diagnostic PCR primer pairs P1 and P2 each use one primer that anneals within the targeting vector and a second primer that is outside the homology region to specifically amplify DNA that has incorporated the targeting vector. C, *IDH1* R132 gDNA sequencing shows that the targeting vector disrupted the *IDH1*^{WT} allele and not the *IDH1*^{R132H} allele in KO-1 and KO-2. The sequence of PCR product P3 reflects the genotype of both the intact and targeted alleles, whereas P4 reflects the genotype of the intact allele only. D, cDNA sequencing confirms that KO-1 and KO-2 only express *IDH1*^{R132H} and not *IDH1*^{WT}.

glioblastoma, including chromosomes 9q, 10p, 19q or in the genomic regions containing *EGFR*, *PDGFR*, and *PTEN*. IMA cells formed colonies from single cells when single cell diluted and had a doubling time of about 72 hours. The cell line expressed Nestin but was negative for GFAP, Sox2, and Tuj1 and had a stellate morphology (Fig. 2B). As expected for a cell line with an *IDH1* mutation, IMA had more than 80-fold elevated D-2HG than 3 *IDH1* wild-type cell lines (Fig. 2C and Supplementary Fig. S2).

D-2HG production in IDH1-mutated cells requires IDH1^{WT}

To study the effects of loss of the wild-type allele of *IDH1*, we used a recombinant adeno-associated virus targeting system (15) to knock out one *IDH1* allele in IMA (Fig. 3A). After screening 1,034 clones, we obtained 11 positive clones with PCR-confirmed homologous integration into one allele of the native *IDH1* locus (Fig. 3B). To determine which *IDH1* allele (mutant or wild-type) was disrupted in these clones, a region containing the integrated targeting vector and the adjacent *IDH1-R132* locus was PCR amplified and sequenced to determine *IDH1* mutation status. All 11 positive clones disrupted the *IDH1*^{WT} allele and produced only *IDH1*^{R132H} cDNA (*IDH1*^{R132H/-}; Fig. 3C and D).

We focused on 2 *IDH1*^{R132H/-} subclones, KO-1 and KO-2. The morphology, *TP53* and *ATRX* mutation status, and Nestin/Sox2/GFAP/Tuj1 status of *IDH1*^{R132H/-} cells was indistinguishable from the parental IMA cells. No growth defect was observed for *IDH1*^{R132H/-} cells, and the percentage of apoptotic cells was less than 2% in *IDH1*^{R132H/-} and parental cells (Supplementary Fig. S2A and S2B). Compared with parental IMA (*IDH1*^{R132H/WT}), D-2HG was 87-fold lower in those 2 *IDH1*^{R132H/-} subclones (Fig. 2C). Ectopic re-expression of

IDH1^{WT} in KO-1 (*IDH1*^{R132H/-}) cells rescued D-2HG levels to 52-fold higher than an empty vector control (Fig. 4A and B).

To confirm these findings in a second cell line, we investigated the colorectal carcinoma cell line HCT116 (13). Knock-in of an *IDH1*^{R132H} allele to produce heterozygous *IDH1*^{R132H/WT} (13) resulted in more than 100-fold higher D-2HG than the parental *IDH1*^{WT/WT} HCT116 cells. We were also able to obtain an *IDH1*^{R132H/WT} subclone of HCT116 in which allelic variation occurred such that only the *IDH1*^{R132H} allele, but not the *IDH1*^{WT} allele, was expressed on the basis of cDNA sequencing and immunoblot with *IDH1* and *IDH1*^{R132H} antibodies (Supplementary Fig. S3A and S3B). In contrast to the HCT116 cells expressing both mutant and wild-type *IDH1* alleles, the subclone expressing *IDH1*^{R132H} alone did not exhibit any elevation of D-2HG levels (Supplementary Fig. S3C). Thus, both wild-type and mutant *IDH1* alleles are necessary to achieve elevated D-2HG levels in 2 cancer cell lines, IMA and HCT116.

We hypothesized that 2 mechanisms could contribute to a requirement for *IDH1*^{WT} to overproduce D-2HG: (i) knocking out *IDH1*^{WT} impairs the conversion of isocitrate to α KG, the substrate for D-2HG production, leading to intracellular α KG levels that are too low for efficient conversion to D-2HG or (ii) efficient D-2HG production requires the heterodimer formed by wild-type and mutant *IDH1*. To exclude the first possibility, we incubated KO-1 (*IDH1*^{R132H/-}) cells with cell-permeable octyl- α KG and measured the cellular concentrations of α KG and D-2HG. Without octyl- α KG treatment, the KO-1 cells had slightly lower α KG levels than the parental IMA cells, but restoring cellular α KG to the α KG levels in the parental IMA cells could not rescue D-2HG production in the KO-1 cells (Fig. 4C). This finding suggests that the wild-type:mutant *IDH1* heterodimer is a major mediator of D-2HG production in glioma cells.

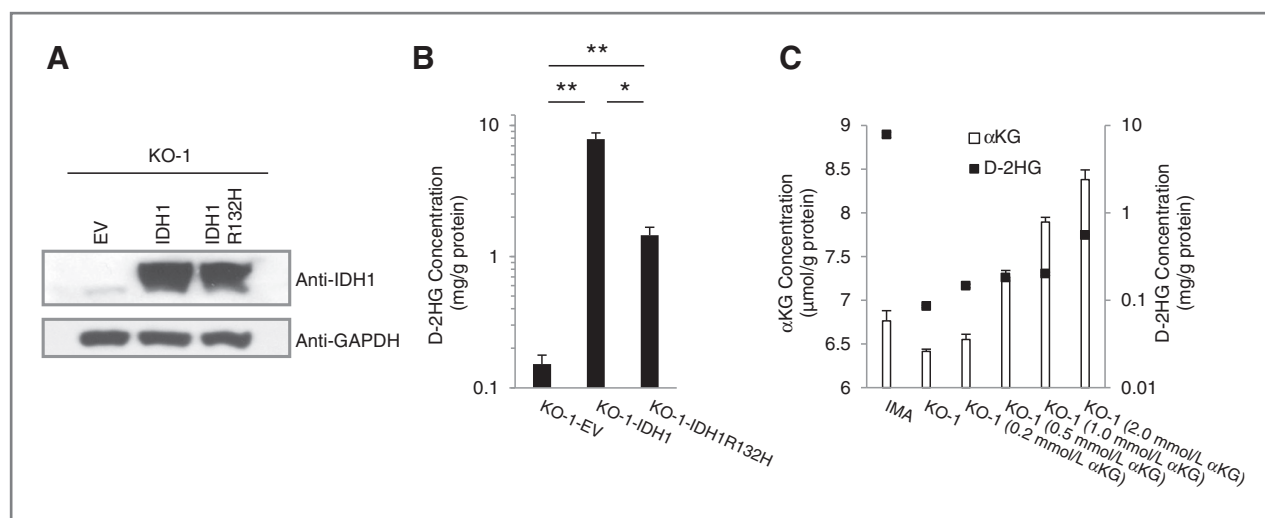


Figure 4. Efficient D-2HG production in IDH1 mutant cells requires wild-type IDH1. A, KO-1 (*IDH1*^{R132H/-}) cells were infected with lentivirus expressing wild-type *IDH1*^{WT} or *IDH1*^{R132H}. After 1 month of selection, cell lysates were prepared for Western blotting using cells infected with empty vector (EV) lentivirus as a control. B, D-2HG levels in lysates from indicated KO-1 (*IDH1*^{R132H/-}) cells with ectopic expression of vector control, wild-type *IDH1*^{WT}, or *IDH1*^{R132H}. C, KO-1 (*IDH1*^{R132H/-}) cells were treated with the indicated concentrations of cell permeable octyl- α KG for 20 hours, followed by measurement of cellular α KG and D-2HG levels. Mean \pm SD are shown for samples analyzed in triplicate and are representative of 2 independent experiments. *, $P < 0.05$; **, $P < 0.0001$, Student *t* test.

Discussion

In this report, we characterize IMA, an anaplastic astrocytoma WHO grade III cell line, with *IDH1*^{R132H/WT} mutation. This line joins an anaplastic oligodendroglioma line and an anaplastic oligoastrocytoma line as glioma cell lines that faithfully maintain an *IDH1* mutation from the primary tumor (17, 18). WHO grade II–III astrocytomas were recently found to frequently (>60%) contain *IDH1*, *TP53*, and *ATRX* co-mutation (19). IMA provides a model to study the role of these mutations in astrocytoma pathogenesis. IMA exhibited Nestin staining, a marker for neuronal progenitor cells, consistent with *IDH1*-mutated gliomas exhibiting gene expression profiles that are more similar to neuronal progenitor cells (proneural) than other gliomas (20).

The results show that both the wild-type and the mutant *IDH1* alleles are necessary to elicit highly elevated cellular D-2HG levels (>4 mg D-2HG/g protein) in IMA and HCT116. Characterization of two glioblastomas that lost the wild-type *IDH1* allele and had relatively low D-2HG suggests that both wild-type and mutant *IDH1* alleles are also required for high levels of D-2HG production *in vivo*. These results indicate that the wild-type:mutant *IDH1* heterodimer is critical to support faster α KG \rightarrow D-2HG turnover and/or to support "coupled" isocitrate \rightarrow D-2HG conversion in glioma cells, as has been observed for the purified *IDH1* proteins *in vitro* in specific chemical environments (10, 11). Future studies that define the chemical milieu of glioma cells will be needed to determine which chemical conditions, such as the concentration of NADPH or isocitrate substrates, may favor D-2HG production by the wild-type:mutant *IDH1* heterodimer. As modulation of D-2HG levels has been proposed as a potential therapeutic strategy for *IDH1*-mutated tumors, these results warrant a

consideration of the role of wild-type *IDH1* in the development of new treatments.

Disclosure of Potential Conflicts of Interest

Z.J. Reitman has a share of patent on cell lines managed by Duke University. D.D. Bigner shares a license fee through Duke University faculty plan. H. Yan has a commercial research grant from Sanofi-Aventis, honoraria from speakers bureau from Gilead Pharmaceutical and Forma Therapeutics, ownership interest (including patents) in Sanofi-Aventis, Agois Pharmaceuticals, and Personal Genome Diagnostics, Inc., and is a consultant/advisory board member of Sanofi-Aventis. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: G. Jin, Z.J. Reitman, Y. He, H. Yan

Development of methodology: G. Jin, C.G. Duncan

Acquisition of data: G. Jin, C.G. Duncan, I. Spasojevic, B.A. Rasheed, R.E. McLendon, R. Yang

Analysis and interpretation of data: G. Jin, Z.J. Reitman, C.G. Duncan, Y. He, H. Yan

Writing, review, and/or revision of the manuscript: Z.J. Reitman, G. Jin, H. Yan, G.Y. Lopez

Administrative, technical, or material support: R.E. McLendon, D.M. Gooden, D.D. Bigner

Study supervision: H. Yan

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