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Wild-Type IDH1 and Mutant IDH1 Opposingly Regulate Podoplanin Expression in Glioma☆,☆☆

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ABSTRACT

Isocitrate dehydrogenase (IDH) mutations occur frequently in lower-grade gliomas, which result in genome-wide epigenetic alterations. The wild-type IDH1 is reported to participate in lipid biosynthesis and amino acid metabolism, but its role in tumorigenesis is still unclear. In this study, the expressions of IDH1 and podoplanin (Pdpn) were determined in IDH-mutated and IDH-wild-type gliomas, and their relationships in glioma were further analyzed. In addition, the regulation of wild-type IDH1 and mutant IDH1 on Pdpn expression was investigated by luciferase assays and promoter methylation analysis. Our study showed that Pdpn was almost undetectable in IDH-mutated glioma but strongly expressed in higher-grade IDH-wild-type glioma. Pdpn overexpression promoted the migration of glioma cells but had little effect on cell growth. Moreover, Pdpn expression was positively correlated with the increased wild-type IDH1 levels in IDH-wild-type glioma. Consistently, the wild-type IDH1 greatly promoted the transcription and expression of Pdpn, but the mutant IDH1 and D-2-hydroxyglutarate significantly suppressed Pdpn expression in glioma cells. Besides, our results revealed that the methylation of CpG islands in the *Pdpn* promoter was opposingly regulated by wild-type and mutant IDH1 in glioma. Collectively, our results indicated that wild-type and mutant IDH1 opposingly controlled the Pdpn expression in glioma by regulating its promoter methylation, which provides a basis for understanding the relationship between wild-type and mutant IDH1 in epigenetic regulation and tumorigenesis.

Introduction

Glioma is the most common type of primary brain tumors with the median survival of only 12-15 months [1]. In the last decade, isocitrate dehydrogenase (IDH) mutations have been believed to be the most important genetic alteration in glioma. IDH mutations exist in approximately 80% of lower-grade gliomas (LGGs, grade II-III), 85% of secondary glioblastomas (GBMs, grade IV), but only in 5% of primary GBMs [2]. The mutation equips IDH1 with a neomorphic enzyme activity, resulting in a reduction of α-KG into D-2-hydroxyglutarate (D-2-HG) [3]. Since D-2-HG is structurally similar to α-KG, D-2-HG can competitively inhibit α-KG–dependent enzymes [4,5], including histone demethylases and 5-methylcytosine hydroxylases, which are crucial for epigenetic regulation of gene expression [6–9]. IDH mutations cause a global hypermethylated phenotype and gene silencing in glioma, including pyruvate carboxylase, F3, and related vascular genes [10–12], which contribute to the malignancy progression of gliomas.

As a reversible dehydrogenase, wild-type IDH1 is a homodimeric enzyme that reversibly catalyzes isocitrate into α-KG accompanied with the generation of NADPH from $NADP^+$ in cytosol, which has been proven to be physiologically crucial in the metabolism of lipids, amino acids, and

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sugars in cells. In addition, NADPH is an essential cofactor for regenerating the reduced form of glutathione and plays an important role in the progression of maintaining the reduced state of thioredoxin. In primary GBM, wild-type IDH1 was proved to increase the intracellular levels of α-KG, NADPH, and lipid biosynthesis and decrease the ROS level and histone methylation in primary GBM [13], which contribute to the aggressive clinical courses [14,15]. However, the functional relationship between wild-type and mutant IDH1 in tumorigenesis is still elusive.

Podoplanin (Pdpn) is a cell surface membrane glycoprotein carrying a large amount of *O*-glycosides, and it is a mucin-type protein expressed in many tissues [16]. In the neuroepithelium of murine brain, Pdpn regulates the maturation and integrity of the developing vasculature during the embryonic development process [17]. Under physiological conditions, the main function of Pdpn is to induce platelet activation and aggregation by binding to the platelet receptor C-type lectin-like receptor 2 [18]. As an indicator of malignancy, Pdpn has been related with the progression and invasion of various cancers, including GBM [19–23]. Recently, Pdpn is considered as a promising glioma antigen in Car-T therapy [24]. Pdpn expression is controlled by the methylation of its promoter [25], but the crucial mechanism of regulating Pdpn expression is still undefined in glioma. In this study, the roles of wild-type and mutant IDH1 in regulating Pdpn expression were extensively investigated, which might be helpful to understand the malignancy of glioma.

Materials and Methods

Glioma Samples

Glioma tissues were collected from the Department of Pathology at Xijing Hospital, and the Department of Neurosurgery at Tangdu Hospital, the Fourth Military Medical University (FMMU). The sample collection was approved by the Institutional Research Board of FMMU. The glioma samples were classified according to the 2016 WHO classification by experienced neuropathologists, including 29 astrocytomas (WHO II), 28 oligodendrogliomas (WHO II), 21 anaplastic astrocytomas (WHO III), 29 anaplastic oligodendrogliomas (WHO III), and 34 GBMs (WHO IV). IDH1/2 mutations were determined by Sanger sequencing, and 1p/19q co-deletion was detected by fluorescence *in situ* hybridization.

Mice and Primary Glial Cell Culture

Idh1$^{LSL\text{-}R132Q/+}$ mice were a generous gift from Prof. Tak W. Mak, and Gfap-Cre mice (Jax Stock #012886) were purchased from the Jackson Laboratory. *Idh1*$^{LSL\text{-}R132Q/+}$ mice were mated with Gfap-Cre mice to establish *Idh1*$^{LSL\text{-}R132Q/+}$ Gfap-*Cre* (*Idh1*-KI) mice and the corresponding control *Idh1*$^{+/+}$ Gfap-*Cre* (*Idh1*-WT) mice. Mice were housed under standard conditions and maintained under a 12-hour light/12-hour dark cycle with free access to water and food. All experiments involving animals were approved by Institutional Animal Care and Use Committee guidelines of FMMU.

Primary glial cells were isolated from the brains of newborn *Idh1*-KI and *Idh1*-WT mice. Briefly, the meninges and large blood vessels were carefully removed, and the cleaned cerebral cortices were pooled and cut into small pieces. The glial cells were dissociated with Trypsin-EDTA (0.25%), and dissociated cells were resuspended in 0.4 mg/ml DNase. Then, the isolated cells were allowed to attach and grow in 75-cm flasks or six-well plates in DMEM with 10% FBS. The glial cells were harvested from confluent cell monolayers (reached 90%) by shaking cell flasks for 24 hours at 250 rpm.

The Cancer Genome Atlas (TCGA) Database Analysis

In order to evaluate the mRNA levels of the IDH1 and Pdpn in gliomas, we collected data from TCGA (http://www.cbioportal.org) [26,27]. Comparison of mRNA levels between IDH-mutated and IDH-wild-type gliomas was calculated using two-tailed Student's *t* test. The correlation between *Pdpn* and *IDH1* mRNA level was analyzed by linear regression analysis and Pearson's test.

Transwell Migration Assays

Briefly, 1×10^5 U251 or U87 glioma cells in which Pdpn was overexpressed were planted into the apical chambers of an insert (8-μm pore size, Corning) in serum-free medium, and the cells with empty vector (EV) were used as control. Then, DMEM (20% FBS) was added into the basolateral chamber. Following incubation at 37°C for 24 hours, the inserts were cleaned, and 4% paraformaldehyde was used to fix the invaded cells. After staining with 0.1% crystal violet dye, the migrated cells were numbered.

Intracranial Glioma Xenografts

Five- to 6-week-old nude mice on a BALB/c background were purchased from the Experimental Animal Centre of the FMMU. The nude mice were anesthetized, and U87 glioma cells (5×10^5 cells per mouse) with overexpressed Pdpn were stereotactically inoculated into the right side of the brain 2 mm lateral to the bregma and 3 mm below the dura matter. Seven days after inoculation, the mice were anesthetized with 4% chloral hydrate and fixed by heart perfusion with 4% paraformaldehyde. Then their brains were dissected, and the intracranial glioma xenografts were analyzed by hematoxylin and eosin (HE) staining and Ki-67 immunohistochemical staining.

Cell Proliferation Determination

The cells were planted in a 96-well plate and stained with cell counting kit-8 (CCK8) solution, and the absorbance reading was taken at 450 nm with a microplate reader (Multiskan Go, Thermo Fisher).

Pdpn Promoter Methylation Analysis

Paraffin-embedded glioma tissues were collected to extract genomic DNA using a FFPE DNA Kit (AmoyDx, China), and 1-2 μg extracted genomic DNA was treated with sodium bisulfite (the Epitect Fast DNA Bisulfite Kit Qiagen) in line with the instructions. The CpG islands in the *Pdpn* promoter were amplified as follows: forward, 5′-GCG GCC GCG TTG AGT AGA ATA AAA GTT TAT TTT GTT AGG-3′ and reverse, 5′-CAA CAA ACT TTA CAA TTA AAA ACT TTC-3′. The 243-bp amplified fragments were purified using a DNA purification kit (Qiagen), and the methylation of CpG islands in the *Pdpn* promoter was analyzed after Sanger sequencing and calculated using the C/T ratio.

Immunohistochemical Staining

Immunohistochemistry was performed using standard protocols. The primary antibodies included mouse anti-human IDH1 (R132H) (MAB-0733, MXB Biotech), rabbit anti-human IDH1 (8137, Cell Signaling Technology), and mouse anti-human Pdpn (D2–40, MXB Biotech). IHC score was assigned to each case *via* light microscopy in accordance with the following intensity: 0 (negative), 1 (weak), 2 (moderate), and 3 (strong staining).

Luciferase Reporter Assays

The human *Pdpn* promoter fragment (−857/+205) was amplified from genomic DNA with the primers 5′-GCG AAG CTT ACG TAA GTT CTC TGG TTG CTG A-3′ and 5′-TCA CCA TGG TTC CCG TTG AGT TGT TGC TCT C-3′. The products of polymerase chain reaction (PCR) were cloned into the pGL3-basic vector (Promega) between the *Hind*III and *Nco*I restriction sites. Then 293T cells were transfected with the different

expression plasmids using polyethylenimine (Sigma-Aldrich). In each well of 24-well plates, 20 ng of the Renilla luciferase vector pRL-TK (Promega) was chosen as the internal reference for transfection efficiency, and pGL3-basic served as a negative control. The pGL3-*Pdpn* promoter constructs were co-transfected with 400 ng of pcDNA3.1-IDH1-WT or pcDNA3.1-IDH1-R132H into 293T cells to investigate the effects of wild-type and mutant IDH1 on *Pdpn* transcription. In addition, 293T cells transfected with the pGL3-*Pdpn* promoter constructs were treated with Octyl-2HG (gift from Prof. Qingxi Li at Xiamen University, China) or D-2-HG (S7873, Selleck) to evaluate the effects of 2-HG on *Pdpn* transcription. After 12 hours, these cells were harvested and measured with Dual-Luciferase Reporter Assay System (Promega) to detect Renilla luciferase activities. All samples were assayed in quadruplicate, and measurements were carried out using Lumat LB 9507 luminometer (EG&G Berthold).

Lentivirus Generation and Infection

Full-length *Pdpn* (NM_006474.4) was subcloned into the *AsiS*I and *Xho*I restriction sites of the lentiviral vector pLenti-C-Myc-DDK-IRES-Puro (Origene), and the sequence was confirmed by Sanger sequencing. The constructed product was co-transfected into HEK-293T cells with lentiviral packaging plasmids (pAX2 and pVSVG), and the lentiviruses were harvested at 72 hours after transfection. After filtering through 0.45-μm filters, the reconstructed lentiviruses were concentrated through ultracentrifugation (2 hours at 50,000 × *g*) and purified on a gradient (20% sucrose, 2 hours at 46,000 × *g*) as previously described [28]. Then the reconstructed lentiviruses were used to infect U87 and U251 cells for 72 hours, and 3-8 μg/ml puromycin was chosen to select the infected cells. Pdpn protein levels in these cells were determined by immunoblotting.

To express the wild-type and mutant IDH1 in U87 and U251 cells, the wild-type IDH1 and mutant IDH1 (R132H) were subcloned into the pLenti-C-Myc-DDK-IRES-Puro vector (Origene) and pRRLSIN-cPPT-CMV-IRES-Hygro vector (Eureka Biotech, China), respectively, and the lentiviruses were generated and purified as previously described [29]. After being infected, the U87 and U251 cells were selected with puromycin and/or hygromycin.

Quantitative PCR (qPCR)

TRIzol (Invitrogen) was used to extract total RNA from tissues and cells. The extracted RNA was reverse-transcribed into cDNA with Primer Script RT Reagent kit (TaKaRa, Japan). After quantification, the cDNA was subsequently subjected to ABI PRISM 7500 system (Applied Biosystems) to perform qPCR using SYBR Green kit (TaKaRa, Japan). The primers are presented in Supplementary Table 1.

Immunoblotting

The total protein was extracted using RIPA buffer from the cell and tissue samples and quantified by Bradford method. Equal amounts of protein (40 μg/lane) were separated using SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA). PBST (5% skim milk) was used to block polyvinylidene difluoride membrane for 1 hour and then incubated with respective primary antibodies overnight at 4°C. After washing with PBST, the membranes were subsequently

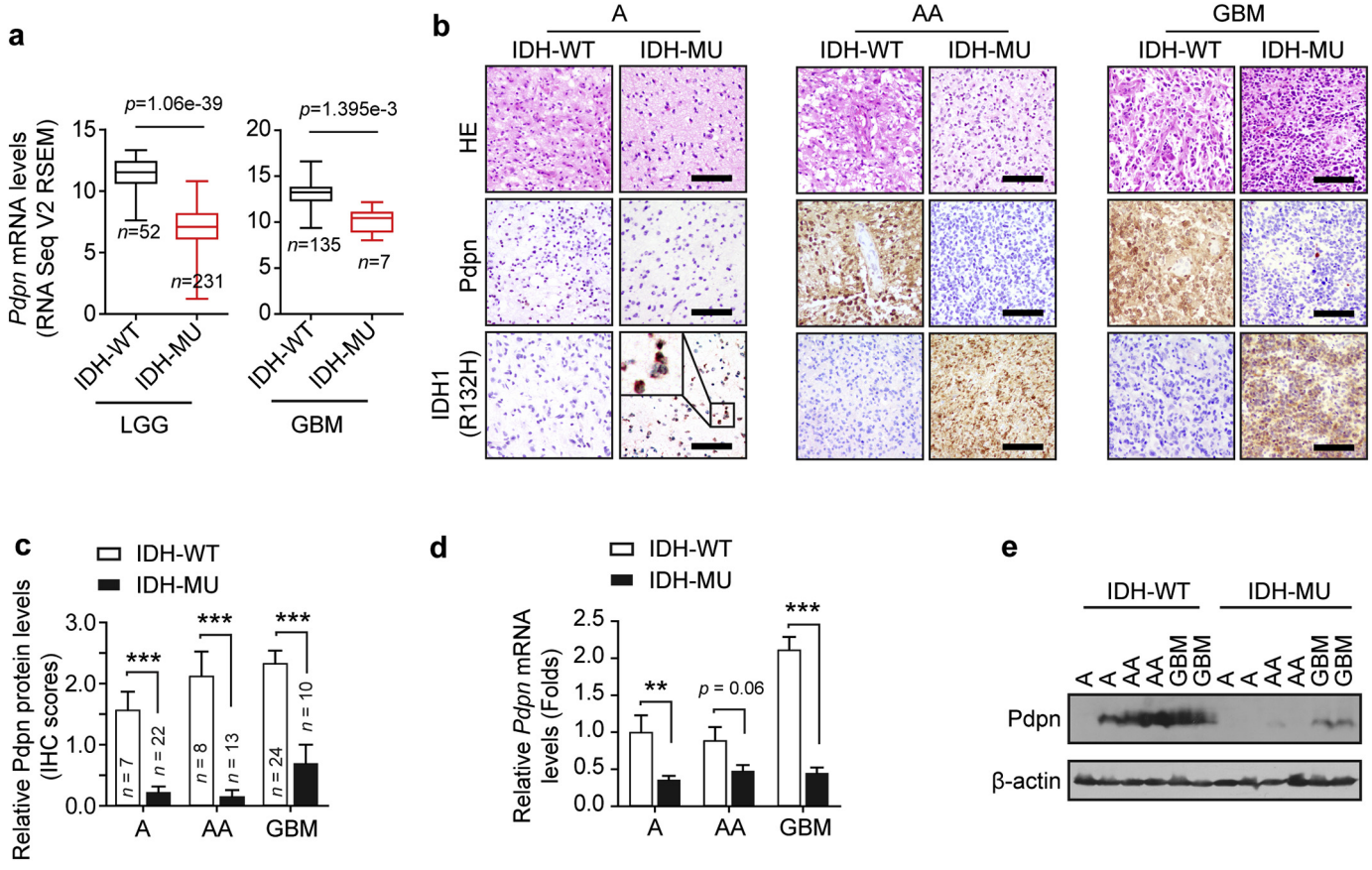

Figure 1. The expression of Pdpn was significantly reduced in IDH-mutated glioma. (A) The mRNA levels of *Pdpn* were obtained and analyzed using TCGA LGG and GBM datasets. (B) The expression of Pdpn and IDH1 (R132H) was detected by IHC staining in astrocytoma (A), anaplastic astrocytoma (AA), and GBM with mutant (IDH-MU) and wild-type (IDH-WT) IDH. Bar = 100 μm. (C) The immunoreactivity of Pdpn in the specimens was scored: 0 (negative), 1 (weak), 2 (moderate), and 3 (strong staining) (D) The mRNA levels of *Pdpn* in IDH-MU and IDH-WT gliomas (n = 6 in each group) were analyzed by qPCR. *P < .05, **P < .01, ***P < .001. (E) Immunoblotting to detect Pdpn protein in IDH-MU and IDH-WT gliomas (A, AA, and GBM).

incubated with proper secondary antibodies for 1 hour at room temperature. After the membranes were extensively washed, the signals were detected by enhanced chemiluminescence and exposure to X-ray film. The primary antibodies used in this study are as follows: mouse anti-human Pdpn (D2-40, MXB Biotech, 1:100), golden Syrian hamster anti-mouse Pdpn (8.1.1, EBioscience, 1:1000), mouse anti-human IDH1 (R132H) (MAB-0733, MXB Biotech, 1:1000), goat anti-human IDH1 (sc-49996, Santa Cruz, 1:1000), mouse anti-human β-tubulin (KM9003T, Sungene Biotech, 1:1000), and mouse anti-human β-actin (KM9001, Sungene Biotech, 1:1000).

α-KG Measurement

The glioma tissues, cells, and mice brains were deproteinized with perchloric acid. Intracellular levels of α-KG were determined by commercial α-ketoglutarate Assay kit (ab83431, Abcam) according to the instructions.

Statistical Analysis

All results represent the mean ± standard error (SE). Statistical analysis was performed using the Student's *t* tests by SPSS 13.0 software (SPSS, USA). *P* values smaller than .05 were considered as statistically significant.

Results

Pdpn Is Significantly Reduced in IDH-Mutated Glioma

It is reported that high Pdpn expression is associated with worse prognosis in glioma [20]. The TCGA database indicated that *Pdpn* mRNA levels were significantly decreased in IDH-mutated LGG (P = 1.06e-39) compared with IDH-wild-type LGG, and a similar tendency was observed in GBM (P = 1.395e-3) (Figure 1*A*). Consistent with these results, the immunohistochemical staining also showed that Pdpn was greatly reduced in astrocytoma (A), anaplastic astrocytoma (AA), and GBM with mutant IDH (IDH-MU) compared to the same subtypes with wild-type IDH (IDH-WT) (Figure 1*B*). Also, the statistical analysis results confirmed this finding (Figure 1*C*). What's more, the same results were observed in oligodendroglioma (O) and anaplastic oligodendroglioma (AO) (Figure S1). In addition, qPCR and immunoblotting showed that Pdpn expression was notably higher in GBM compared with LGG but was suppressed by IDH mutations in all astrocytic tumors, including A, AA, and GBM (Figure 1, *D* and *E*). Collectively, these data suggested that IDH mutations reduced Pdpn expression in glioma.

Pdpn Overexpression Promotes the Migration and Infiltration of Glioma

To ascertain the roles of Pdpn in IDH-wild-type glioma, Pdpn was overexpressed in U87 and U251 cells by infection with lentivirus. Our results indicated that the Pdpn overexpression did not affect the expression of proliferating cell nuclear antigen (Figure 2, *A* and *B*). Consistently, CCK8 assay indicated that Pdpn overexpression had no obvious effect on the growth of U87 and U251 glioma cells. However, the Transwell assay showed that Pdpn overexpression promoted the migrations of U87 and U251 glioma cells *in vitro* (Figure 2, *C* and *D*), which was consistent with the previous report [30]. Furthermore, the U87 intracerebral orthotopic implant model was used to further investigate the functions of Pdpn overexpression in IDH-wild-type glioma. HE staining showed that Pdpn overexpression increased the infiltrations of U87 glioma cells, but IHC staining indicated that Pdpn overexpression did not increase the proportion of

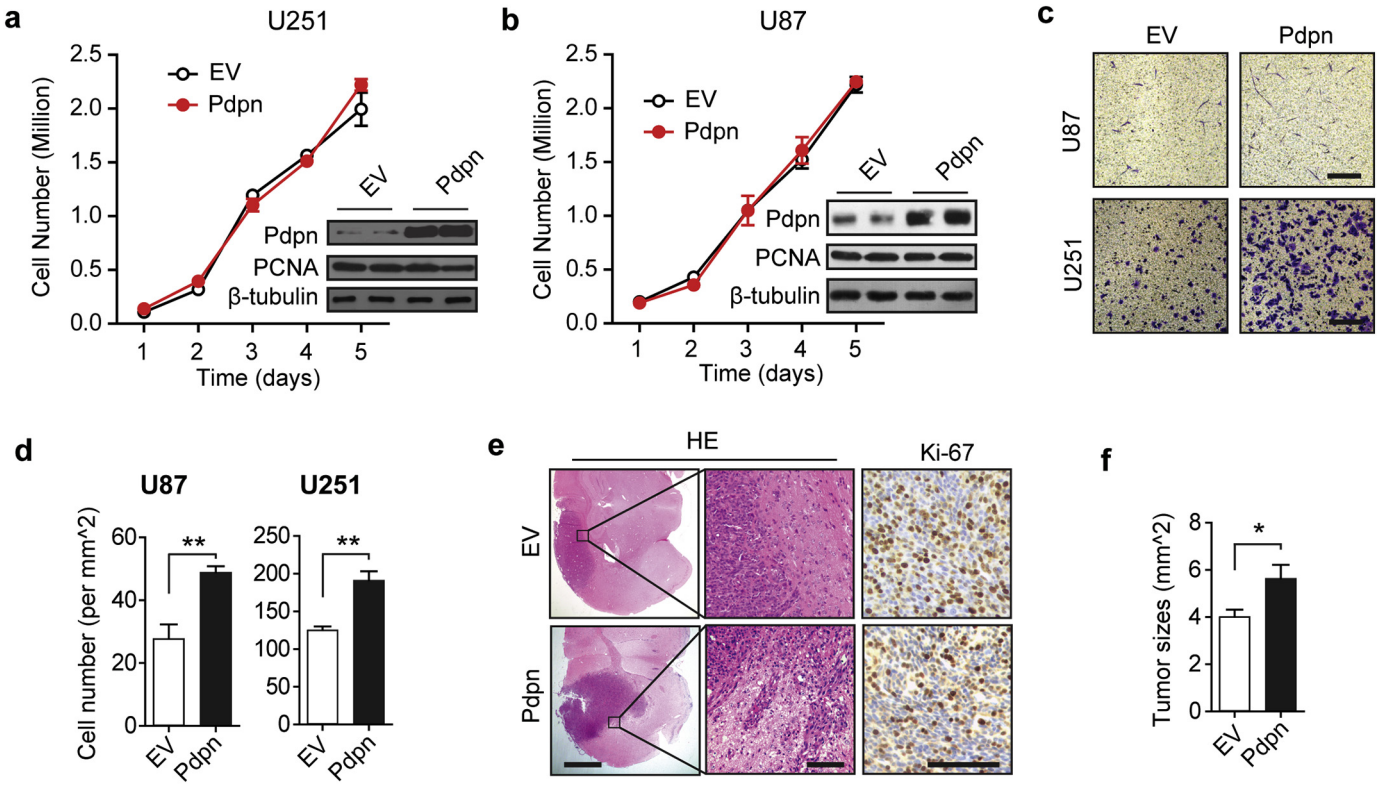

Figure 2. Pdpn overexpression promoted the infiltration of glioma cells. Pdpn was overexpressed in U251 and U87 cells by lentivirus infection, and the lentivirus with EV was used as control. (A) The expressions of Pdpn and proliferating cell nuclear antigen were detected by immunoblotting. (B) The growth of U251 and U87 cells overexpressing Pdpn was analyzed by CCK8 methods. (C) The migrations of U251 and U87 cells overexpressing Pdpn were determined by Transwell assay. (D) Statistical analysis of the effects of Pdpn overexpression on the migration ability of U87 and U251 glioma cells. Bar = 200 μm, $^*P < .05$, $^{**}P < .01$. (E) Nude mice received intracerebral implantation of Pdpn-overexpressed U87 cells. The infiltration of glioma cells was observed under HE staining, and the proportion of Ki-67 labeled cells was analyzed by IHC staining. Bar = 1 mm (left) or 100 μm (middle and right). (F) The sizes of U87 xenograft with or without Pdpn overexpression were analyzed statistically.

Ki-67–labeled cells (Figure 2*E*). Moreover, the volume of U87 xenograft with Pdpn overexpression was significantly larger than that of control xenograft with EV (Figure 2*F*). These results indicated that Pdpn overexpression promoted the infiltration of glioma without affecting the cell proliferation.

Pdpn Is Remarkably Upregulated in IDH-Wild-Type Glioma and Positively Related to IDH1 Levels

Pdpn is undetectable in normal brain tissues, but it was observed in the glioma tissues. Therefore, we investigated the regulatory mechanisms of the increased Pdpn expression in IDH-wild-type glioma. The TCGA datasets showed that the levels of *Pdpn* mRNA were elevated and positively correlated with the *IDH1* mRNA levels in IDH-wild-type glioma (Figure 3*A*). The qPCR results also confirmed that the levels of *Pdpn* mRNA were elevated in IDH-wild-type glioma and positively correlated with *IDH1*mRNA in IDH-wild-type glioma, but *Pdpn* mRNA was obviously reduced and had no correlation with *IDH1* mRNA levels in IDH-mutated glioma (Figure 3*B*). Interestingly, qPCR and immunoblotting results indicated that the mRNA and protein levels of total IDH1 (wild-type and mutated) were comparable in both IDH-wild-type and IDH-mutated gliomas but increased with increasing tumor grade (Figure 3, *C* and *D*). In addition, IHC staining showed that the total IDH1 (wild-type and mutated) protein levels were significantly increased in GBM with or without IDH mutations when compared with the peritumor normal brain tissues (Figure 3*E*). Taken together, our results suggested that IDH1 was elevated in glioma, and wild-type IDH was positively correlated with the transcription and expression of Pdpn in IDH1-wild-type glioma.

Wild-Type IDH1 Greatly Promotes Pdpn Expression in Glioma Cells

To further examine whether the wild-type IDH1 could regulate the expression of Pdpn in glioma, the wild-type IDH1 (wtIDH1) was overexpressed in U251 and U87 glioma cells by lentivirus infection, and the lentiviruses with EV were used as controls. Immunoblotting and qPCR indicated that wild-type IDH1 overexpression significantly increased the expression of Pdpn in both U87 and U251 cells (Figure 4, *A* and *B*). Moreover, the relative luciferase activity was measured to detect the effect of wild-type IDH1 on *Pdpn* promoter transcription. As described in the methods, the human *Pdpn* promoter fragment (−857/+205) was cloned into the pGL3-basic vector, and the luciferase activity in 293T cells demonstrated that wild-type IDH1 substantially elevated the transcriptional activity of *Pdpn* promoter compared to the controls (EV) (Figure 4*C*). Immunoblotting showed that IDH1 deficiency decreased Pdpn expression in brain and primary glia cells compared with control wild-type mice (Figure 4*D*). In summary, these findings indicated that wild-type IDH1 greatly promoted Pdpn expression in glioma cells by increasing the transcriptional activity of the *Pdpn* promoter.

Both Mutant IDH1 and D-2-HG Suppress Pdpn Expression in Glioma Cells

To investigate the reduced Pdpn expression in IDH-mutated glioma, the mutant IDH1 (R132H) was overexpressed into U87 and U251 cells with or without overexpressing wild-type IDH1. Immunoblotting showed that the mutant IDH1 significantly inhibited Pdpn expression in both U251 and U87 cells with overexpressed wild-type IDH1 but did not further reduce the Pdpn levels in the parental U251 or U87 cells (Figure 5*A*). Quantitative

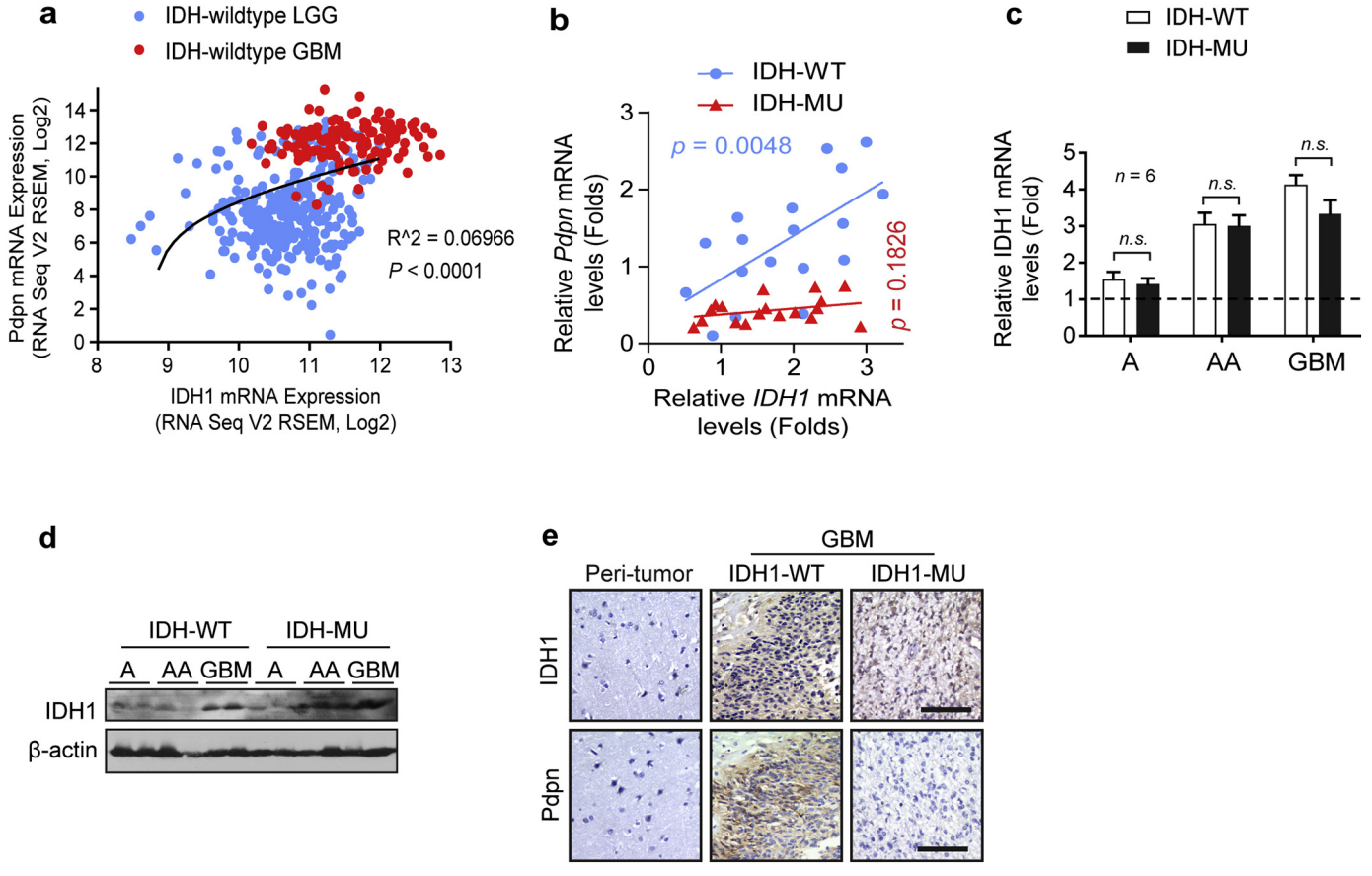

Figure 3. Pdpn expression was remarkably upregulated in IDH-wild-type glioma and positively correlated with IDH1 levels. (A) TCGA datasets (http://www.cbioportal.org) showed that the *IDH1* mRNA levels in IDH-wild-type GBM were higher than those in IDH-wild-type LGG, and both were correlated with Pdpn levels. (B) Correlation analysis was performed between total IDH1 (mutant and wild-type) and *Pdpn* mRNA levels in IDH-MU and IDH-WT gliomas. (C and D) IDH1 expression in IDH-MU and IDH-WT gliomas was analyzed by qPCR (C) and immunoblotting (D). (E) IHC staining showed that the total IDH1 (wild-type and mutant) was elevated in both IDH-wild-type (IDH-WT) and IDH-mutated (IDH-MU) GBM compared with the peritumor tissues, but the Pdpn protein levels were significantly increased only in IDH-WT GBM. Bar = 100 μm.

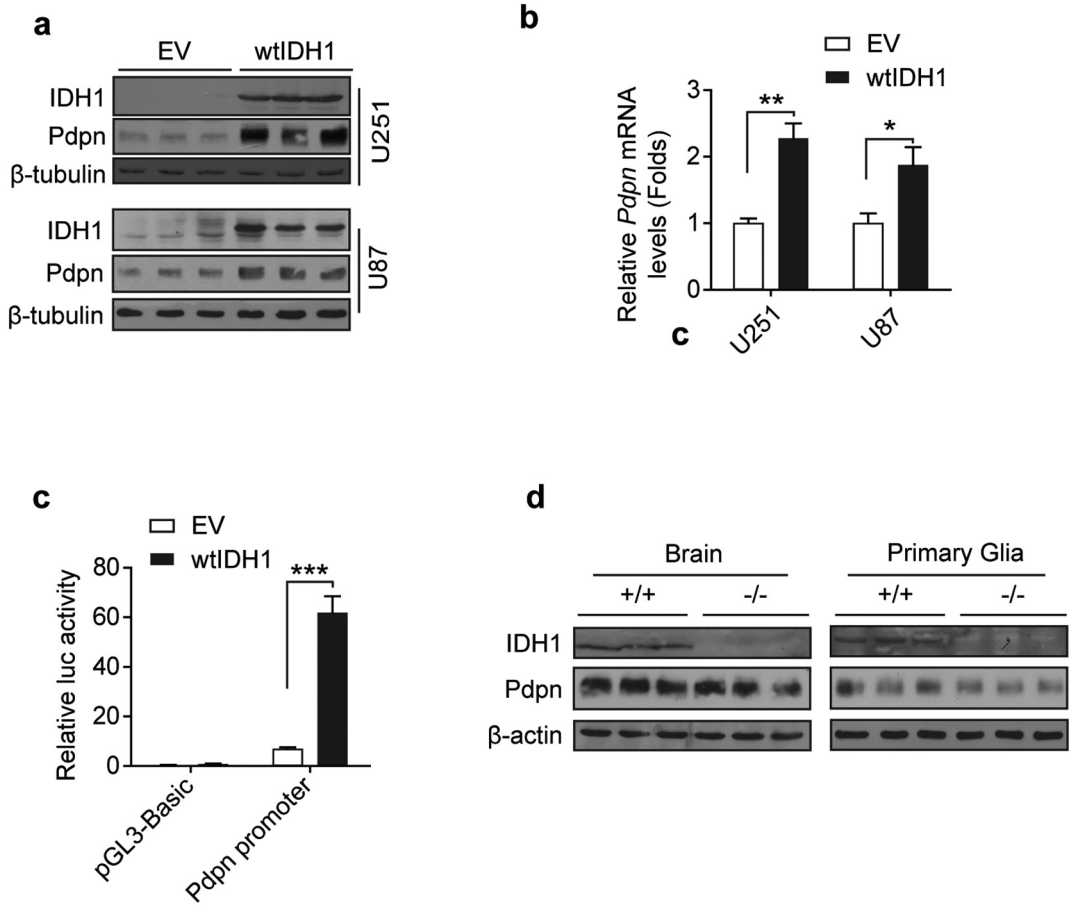

Figure 4. Wild-type IDH1 greatly promoted Pdpn expression in glioma cells. Wild-type IDH1 (wtIDH1) was overexpressed in U251 and U87 glioma cells by lentivirus infection, and lentivirus with EV were used as controls. (A and B) The expression of IDH1 and Pdpn in U87 and U251 cells was analyzed by immunoblotting (A) and qPCR ($n = 6$) (B). (C) The human *Pdpn* promoter fragment ($-857/+205$) was cloned into the pGL3-basic vector, and the luciferase activity was determined in 293T cells with or without overexpressing wtIDH1. (D) Pdpn expression was measured in brain and primary glia of *Idh1* knockout ($Idh1^{LSL/LSL}$, $-/-$) and wild-type ($+/+$) mice. The results are representative of two or three independent experiments. The data are presented as the mean ± SE ($n = 4$). * $P < .05$, ** $P < .01$, and *** $P < .001$.

PCR also showed similar results (Figure 5*B*). Moreover, the luciferase assay indicated that mutant IDH1 inhibited the elevated transcriptional activity of the *Pdpn* promoter in U87 and U251 cells with wild-type IDH1 overexpression (Figure 5*C*). Mutant IDH produces D-2-HG, which functions as an oncometabolite in glioma cell. To test the impact of D-2-HG on *Pdpn* transcription activity and expression, the isolated primary glial cells and wild-type IDH1 overexpressed U251 cells were treated with D-2-HG or octyl-2-HG (cell permeable), respectively. Immunoblotting and qPCR showed that both D-2-HG and octyl-2-HG reduced Pdpn protein and mRNA levels in the isolated primary glial cells in a dose-dependent manner (Figure 5, *D* and *E*). Consistent with these findings, both octyl-2-HG and D-2-HG decreased Pdpn protein levels in U251 cells overexpressing wild-type IDH1 (Figure 5*F*). Furthermore, the luciferase assay results indicated that both D-2-HG and octyl-2-HG inhibited the transcriptional activity of the *Pdpn* promoter in 293T cells (Figure 5*G*). In conclusion, these results implied that both D-2-HG and IDH1 mutation in glioma downregulated the expression of Pdpn, which was opposite with the effect of wild-type IDH1.

The Methylation of the Pdpn Promoter Is Opposingly Regulated by Wild-Type and Mutant IDH1 in Glioma

The transcription of *Pdpn* mRNA is controlled by the methylation of its promoter. Here, we found that the CpG islands in the *Pdpn* promoter were hypermethylated in IDH-mutated gliomas but significantly demethylated in IDH-wild-type gliomas when compared with the peritumor tissues (Figure 6*A*), which were consistent with the expression of Pdpn. In U251 glioma cells, the wild-type IDH1 overexpression vastly demethylated the CpG islands in the *Pdpn* promoter, while mutant IDH1 (R132H) expression restored the hypermethylation of the *Pdpn* promoter (Figure 6*B*). The demethylation of *Pdpn* promoter by wild-type IDH1 could not be explained by the increase in D-2-HG, which is only produced by mutant IDH. Both wild-type and mutant IDH1 can catalyze the metabolism of cytosolic α-KG, a co-substrate of DNA demethylation. Therefore, we demonstrated that the α-KG levels in IDH-mutated glioma were lower than those in IDH-wild-type glioma (Figure 6*C*), and the knockin of mutant IDH1 (R132H) consistently reduced the intracellular α-KG levels in the mice primary glia cell (Figure 6*D*). *In vitro*, wild-type IDH1 elevated the intracellular α-KG in U251 glioma cells, but the mutant IDH1 reduced the intracellular α-KG levels (Figure 6*E*). In addition, we found that both octyl-2-HG and D-2-HG consistently hypermethylated the CpG islands in the *Pdpn* promoter in U251 cells overexpressing wild-type IDH1 (Figure 6*F*). In summary, the wild-type and mutant IDH1 opposingly regulated the intracellular α-KG levels to modulate the patterns of *Pdpn* promoter methylation.

Discussion

IDH mutation is perceived as an important principle in glioma classification, especially in LGG. Compared to other tumors, IDH mutation in glioma has a unique effect on DNA methylome and transcriptome, which could explain why it is a favorable prognostic marker only in glioma. The recurrence of G-CIMP-high glioma and the transformation into G-CIMP-low are largely due to the loss of DNA methylation, which is related with the poor

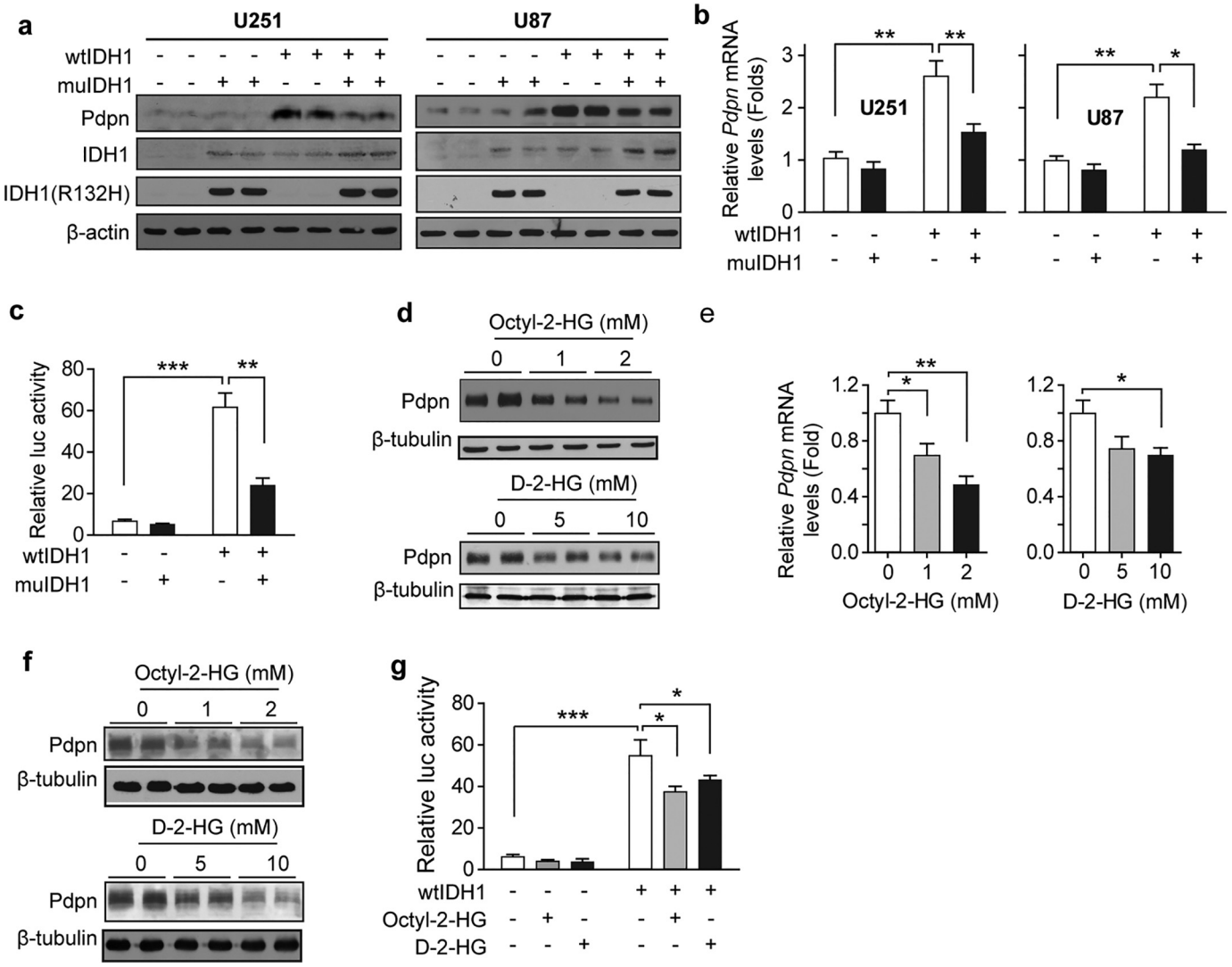

Figure 5. Mutant IDH1 and D-2-HG suppressed Pdpn expression in glioma cells. (A and B) Mutant IDH1 (muIDH1) was overexpressed in U87 and U251 cells by infection with lentivirus, individually or together with wild-type IDH1 (wtIDH1). Immunoblotting (A) and qPCR (B) detected the expression of IDH1, IDH1 (R132H), and Pdpn. (C) The transcriptional activity of the *Pdpn* promoter was analyzed by luciferase assay in 293T cells with wild-type and/or mutant IDH1 overexpression. (D and E) Primary glial cells were treated with different concentrations of Octyl-2-HG (cell permeable) or D-2-HG, and Pdpn expression was determined by immunoblotting (D) and qPCR (E). (F) U251 cells with wild-type IDH1 overexpression were treated with different concentrations of Octyl-2-HG (permeable) or D-2-HG, and Pdpn expression was determined by immunoblotting. (G) The transcriptional activities of the *Pdpn* promoter were determined in 293T cells with or without wild-type IDH1 by luciferase assay in the presence of Octyl-2-HG or D-2-HG. The results are representative of two or three independent experiments. The data are presented as the mean ± SE ($n = 4$). * $P < .05$, ** $P < .01$, and *** $P < .001$.

clinical outcome [31,32]. In addition, IDH-wild-type LGG with the lowered G-CIMP is similar to GBM based on molecular features and clinical behaviors [15,33]. However, the interplay between mutant and wild-type IDH1 in epigenetic modifications and tumourigenesis remains unclear.

As a protein, Pdpn is highly glycosylated and expressed in different cell types of humans and rodents [16]. As an important marker of lymphatic vascular endothelial cells, Pdpn plays a crucial role in the development of lymphatic and blood vessels [34,35]. Moreover, Pdpn is strongly overexpressed in malignant astrocytic tumors and highly predictive for reduced overall survival [20]. The elevated expression of Pdpn leads to the decomposition and change of the extracellular matrix of glioma, inducing platelet aggregation and thus promoting the adhesion, migration, and metastasis of tumor cells [36–38]. In this study, we found that Pdpn expression was greatly decreased in IDH-mutated LGG compared with the IDH-wild-type counterparts, and a similar tendency was also observed in GBM. Interestingly, the wild-type IDH1 significantly increased the transcriptional activity of the *Pdpn* promoter, thereby increasing Pdpn expression. In addition, it has been reported that IDH1 overexpression promotes the proliferation of tumor cells and shortens the survival period of tumor-bearing mice [13]. Moreover, IDH1 overexpression in tumors is reported to promote lipid synthesis, reduce ROS production, and promote DNA demethylation [39,40]. Here, we demonstrated that the increased wild-type IDH1 promotes Pdpn expression, which may be crucial for the malignancy of glioma.

It has been confirmed that methylation of the *Pdpn* promoter CpG islands is a key step in regulating its transcription [25]. The DNA methylation is significantly increased in LGG and secondary GBM, which usually harbors IDH1 mutations. Our results confirmed that the methylation of the *Pdpn* promoter CpG islands was significantly increased in IDH-mutated gliomas but in a demethylation state in IDH-wild-type glioma, which was consistent with Pdpn expression trend in gliomas. *In vitro*, the overexpression of wild-type IDH1 in glioma cells greatly reduced the methylation of the CpG islands in *Pdpn* promoter, while IDH1 mutation promoted the methylation of the CpG islands, which was consistent with the effect of wild-type and mutant IDH1 on Pdpn expression. In addition, we found that D-2-HG increased the methylation levels of CpG islands in

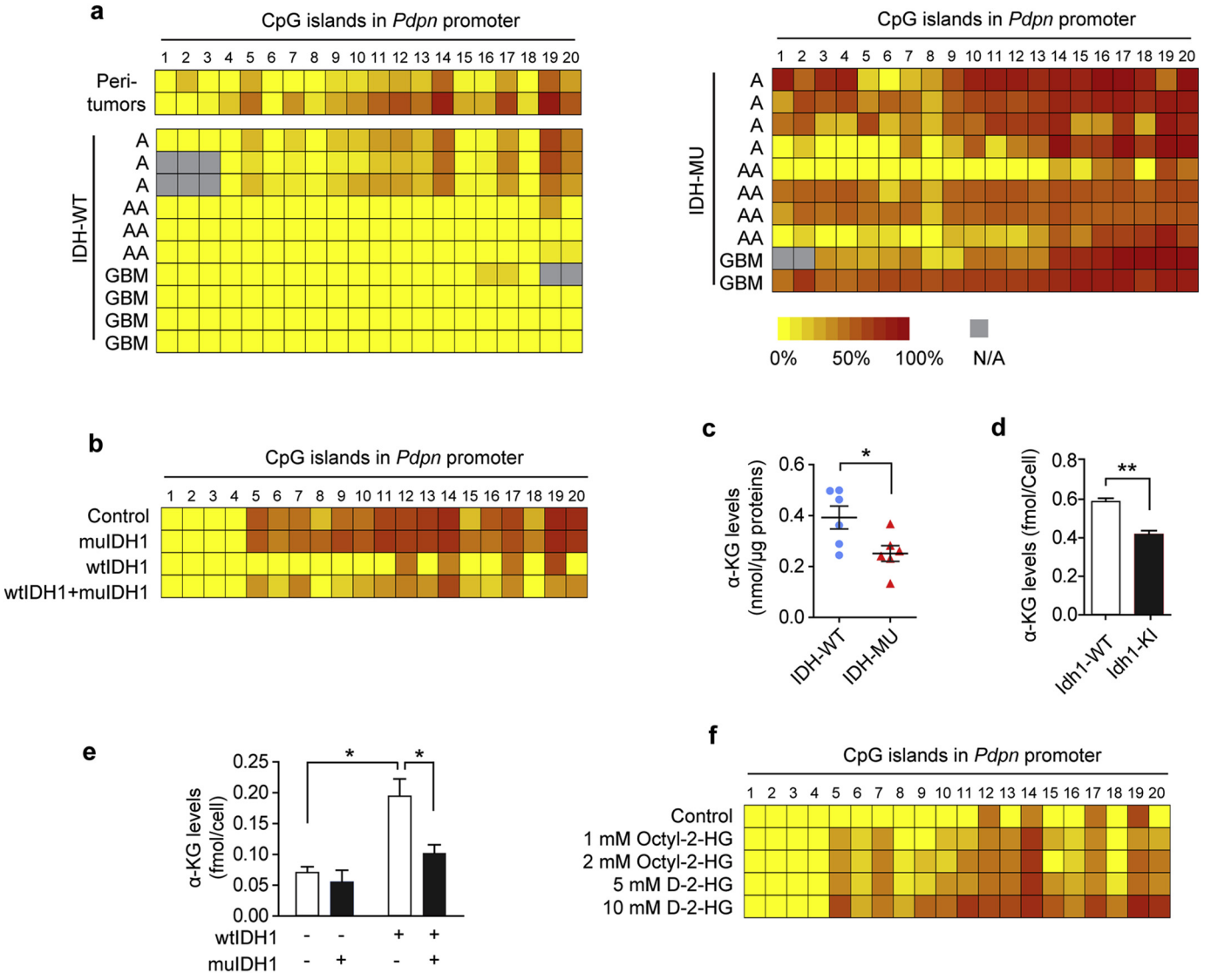

Figure 6. The methylation of the *Pdpn* promoter was opposingly regulated by wild-type and mutant IDH1 in glioma. (A) The methylation of 20 CpG islands in the *Pdpn* promoter was analyzed by bisulfite sequencing in IDH-MU and IDH-WT gliomas compared with peritumor tissues. (B) The methylation of CpG islands in *Pdpn* promoter was evaluated in U251 cells with overexpressing wild-type IDH1 (wtIDH1) and/or mutant IDH1 (muIDH1). (C) The levels of intracellular α-KG in IDH-MU and IDH-WT gliomas ($n = 6$). (D) The α-KG levels in primary glia of mutant IDH1 knockin mice (*Idh1-KI*) and control wild-type mice (*Idh1-WT*). (E) The α-KG levels in U251 glioma cells with overexpressing wtIDH1 and/or muIDH1 ($n = 4$). (F) U251 cells with overexpressed wtIDH1 were treated with different concentrations of Octyl-2-HG or D-2-HG, and the methylation of *Pdpn* promoter was determined by bisulfite sequencing. The results are representative of two or three independent experiments. The data are presented as the mean ± SE. * $P < .05$, ** $P < .01$, and *** $P < .001$.

Pdpn promoter and inhibited Pdpn expression in primary glial cells and glioma cells. These data indicated that IDH1 mutation increased the methylation of the *Pdpn* promoter and thus decreased Pdpn expression, while the wild-type IDH1showed opposite effects (Figure 7).

In mammalian cell, α-KG–dependent dioxygenases are crucial in maintaining the normal physiological functions [41,42]. A recent study revealed that adding α-KG to adult *C. elegans* could greatly extend its lifespan, which was found to be associated with an α-KG binding protein [43]. These findings suggest that the intracellular signaling network controlled by α-KG is more complex than we thought. Furthermore, the tricarboxylic acid cycle produces abundant α-KG, but it is difficult for α-KG to be diffused out of the mitochondria [44]. IDH1 in cytosol can generate higher level of intracellular α-KG [13], which functions as an important co-factor of intracellular dioxygenase to regulate the demethylation of histone and DNA. However, the mutant IDH reduces α-KG to produce D-2-HG, which is believed to be an oncogenic metabolite and competitively inhibits α-KG–dependent dioxygenase activities. Comparing with IDH wild-type

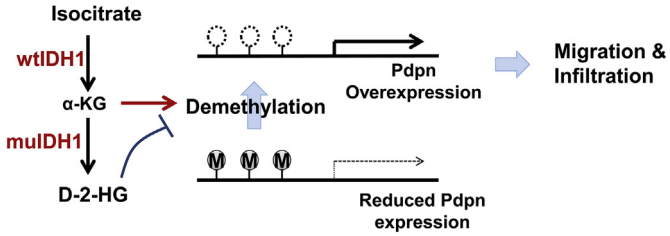

Figure 7. The schematic diagram illustrated the interactive functions of wild-type and mutant IDH1 in glioma. The elevation of wild-type IDH1 (wtIDH1) in glioma cells hypomethylated the CpG island of *Pdpn* promoter by increasing the intracellular α-KG levels, while the mutant IDH (muIDH1) played an opposite function to reduce the intracellular α-KG levels and resulted in the hypermethylations of *Pdpn* promoter. D-2-HG, generated by muIDH1, also directly impeded the demethylation of *Pdpn* promoter at a higher concentration. The hypomethylation of *Pdpn* promoter increased the Pdpn transcription, which stimulated the migration of glioma cells.

glioma, the expressions of PD-L1, transgelin-2 (TAGLN2), and branched-chain amino acid transaminase 1 (BACT1) were also significantly reduced in IDH mutated glioma, which were primarily due to the promoters hypermethylation [45–47]. Contrary to D-2-HG, α-KG is able to promote the expression of BACT1 at high concentration [47]. In this study, we inferred that the change in α-KG level in cells may be an important factor in wild-type and mutant IDH1 jointly regulating *Pdpn* promoter methylation (Figure 7). Thus, our data indicated that the global DNA hypermethylation in CpG islands in IDH-mutated glioma might result from both the elevated 2-HG and decreased α-KG.

In conclusion, our results revealed that wild-type and mutant IDH1 opposingly regulated intracellular α-KG levels to control the expression of Pdpn by affecting its promoter methylation. This study is of substantial importance for understanding the interactive roles of wild-type and mutant IDH1 in glioma as well as for the development of novel therapeutic strategies.

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