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RESEARCH

Expression Pattern of Id Proteins in Medulloblastoma

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Abstract Inhibitor of DNA binding or inhibitor of differentiation (Id) proteins are up regulated in a variety of neoplasms, particularly in association with high-grade, poorly differentiated tumors, while differentiated tissues show little or no Id expression. The four Id genes are members of the helix-loop-helix (HLH) family of transcription factors and act as negative regulators of transcription by binding to and sequestering HLH complexes. We tested the hypothesis that Id proteins are overexpressed in medulloblastoma by performing immunohistochemistry using a medulloblastoma tissue microarray with 45 unique medulloblastoma and 11 normal control cerebella, and antibodies specific for Id1, Id2, Id3, and Id4. A semi-quantitative staining score that took staining intensity and the proportion of immunoreactive cells into account was used. Id1 was not detected in normal cerebella or in medulloblastoma cells, but 78 % of tumors showed strong Id1 expression in endothelial nuclei of tumor vessels. Id2 expression was scant in normal cerebella and increased in medulloblastoma (median staining score: 4). Id3 expression was noted in some neurons of the developing cerebellar cortex, but it was markedly up regulated in medulloblastoma (median staining score: 12) and in tumor endothelial cells. Id4 was not expressed in normal cerebella or in tumor cells. Id2 or Id3 overexpression drove proliferation in medulloblastoma cell lines by altering the expression of critical cell cycle regulatory proteins in favor of cell proliferation. This study shows that Id1 expression in endothelial cells may contribute to angiogenic processes and that increased expression of Id2 and Id3 in medulloblastoma is potentially involved in tumor cell proliferation and survival.

Keywords Medulloblastoma · Id proteins · Id2 · Id3 · Cerebellum

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Introduction

Medulloblastoma is the most common high grade brain tumor in children and accounts for about 20 % of all pediatric brain tumors [1]. Five-year survival rates are higher than they have ever been, but many challenges remain; as children continue to die of disease well after this 5-year period and survivors are too commonly left with adverse life-long complications due to the effects of radiation therapy administered to the developing brain [2]). These complications typically manifest as declines in intellectual function or psychological issues that impair a survivor's ability to achieve their full potential [2]. There is a pressing need to learn more about medulloblastoma biology to identify new therapeutic targets.

There are four inhibitor of DNA binding or inhibitor of differentiation (Id) proteins, which are encoded by different genes (*ID1*, *ID2*, *ID3* and *ID4*). Id proteins are members of the helix-loop-helix (HLH) family of transcription factors [3]. Dimerization of HLH transcription factors mediates

transcriptional activity, but unlike other HLH proteins, Id proteins lack a DNA binding domain, so when an Id protein binds another HLH protein the complex cannot bind DNA and fails to mediate transcription. This makes Id proteins a naturally occurring dominant negative regulator of transcription.

Much of the early work on Id proteins centered on their role in development [4–6], but Id proteins also regulate many essential aspects of tumor biology such as cell proliferation, differentiation, survival, and invasion as well as angiogenesis and have therefore attracted attention as a potential cancer specific target [3, 7, 8]. Increased Id protein expression is typically associated with high-grade tumors [7] and may be due to persistent growth factor stimulation, but epigenetic mechanisms may also be involved [3, 8, 9]. Studies in models of development and neoplasia indicate that Id proteins maintain cells in a poorly differentiated, proliferative state, and while the precise mechanism of Id protein function is not known, Id proteins are considered positive-regulators of the cell cycle, and can modulate transcription of cyclin-dependent kinase inhibitors [10–12]; while Id2, in particular, binds retinoblastoma and may have a regulatory role in G1 progression [3, 13, 14].

Studies of Id protein expression in human cerebellum and medulloblastoma are limited, so we initiated this study to test the hypothesis that Id protein expression is deregulated in medulloblastoma. We addressed this hypothesis by immunostaining a medulloblastoma tissue microarray with 45 tumor and 11 control cerebella using antibodies that specifically recognize all four Id proteins. We also started to explore the role of Id proteins in cell cycle regulation using medulloblastoma cell lines.

Methods

Medulloblastoma Tissue Microarray

Medulloblastoma tissue microarray slides were obtained from the Biopathology Center at the Research Institute at Nationwide Children's Hospital, following a review of the project by the Institutional Review Board and the Children's Oncology Group. The array includes 45 unique medulloblastoma cases and 11 normal cerebella as controls and was prepared using Children's Oncology Group samples that underwent diagnostic workup and expert peer review. Demographic data were available from 44 of the medulloblastoma cases consisting of 28 males and 16 females. The average age of medulloblastoma patients was 7±4 years (median: 6.75 years, range: 1 month to 16 years, 9 months). The 11 normal cerebellar controls comprised 9 males and 2 females. Five of the controls came from patients within the first year of life (23 gestational weeks, 3 days, 7 days, 4 months and 8 months), which is a critical period of cerebellar development, while the remainder were from older patients (5 years, 6 years, 15 years, 16 years, 21 years and 26 years). The array contained between 1 and 8 duplicate areas from represented tumors and normal controls that were randomly spaced throughout the slide.

Immunohistochemistry

The immunohistochemistry procedure was automated on the BondMax IHC system (Leica Microsystems, Bannockburn, IL) using the Id protein specific antibodies described in Table 1. Antigen retrieval was performed as denoted in Table 1 and signal was detected using the Refine Polymer, DAB (Leica Microsystems, Bannockburn, IL). All antibodies were validated using multi-tissue blocks, containing breast, prostate and kidney. Negative controls were obtained by substituting the primary antibody with a Universal Negative Control Serum (Biocare Medical, Concord, CA)

Staining intensity was scored on a four-tier scale: 0, no staining; 1, mild staining; 2, moderate staining; and 3, strong staining. A categorical system was used for the percentage of tumor cells stained: 0, no tumor cells stained; 1, 1 % to 25 %; 2, 26 % to 50 %; 3, 51 % to 75 %; and 4, over 76 % of tumor cells stained. Two observers (CRP, BMK) recorded immunohistochemical findings using a Zeiss light microscope (Thornwood, NY) with consensus achieved on difficult cases. An immunohistochemical staining score was derived as the product of the overall staining intensity and the percentage of tumor cells stained according to the categorical scale. Many tumors were represented multiple times on the array and the highest staining score was reported for these cases.

Cell Culture

Medulloblastoma cell lines UW288, UW426, D341 and D283 were gifts from Dr. Corey Raffel at Nationwide Children's Hospital. All cell lines were cultured in DMEM supplemented with 10 % fetal bovine serum and incubated at 37°C in 5 % CO_2. Stable cell lines over expressing Id2 or Id3 or empty expression vectors as controls (Origene RC205324, RC200583, PS100007, respectively) were generated using Lipofectamine 2000 per the manufacturer's recommendations (Life Technologies, Grand Island, NY).

Proliferation Assay

One thousand cells were plated in each well of a 96 well plate and cultured in DMEM supplemented with 10 % fetal bovine serum and incubated at 37°C in 5 % CO_2. Ten microlitre of AlamarBlue (Life Technologies, Grand Island, NY) was added to each well and fluorescence was measured at the indicated time points using the SpectraMaxM2E plate reader

Table 1 Immunohistochemistry methods and antibodies

Antigen	Vendor	Clone	Dilution	Antigen retrieval
Id1	Abcam, ab66495	2456C1	1:40	EDTA based, pH 9.0 (ER2 (Leica))
Id2	Abcam, ab52093	rabbit polyclonal	1:25	None
Id3	Abcam, ab41834	rabbit polyclonal	1:150	Citrate based, pH 6.0 (ER1 (Leica))
Id4	Abcam, ab20988	rabbit polyclonal	1:300	Citrate based, pH 6.0 (ER1 (Leica))

(Molecular Devices, Sunnyvale, CA). Each experiment was performed three times with four replicates for each time point.

Western Blotting

Cells were lysed using Mammalian Protein Extraction Reagent (Pierce Biochemical Rockford, IL) and protein concentrations determined using the BCA method (Pierce, Rockford, IL). Protein samples were separated through 4–20 % Tris-Glycine gels (Invitrogen), transferred to PVDF membranes (Biorad, Hercules, CA), and probed with appropriate antibodies (Table 2). Immun-Star Western C (Biorad, Hercules, CA) chemiluminescent substrate was applied to the membranes, which were imaged using a VersaDoc (Biorad, Hercules, CA). Densitometric analysis was performed using contour analysis in ImageLab software v.2.0.1 (Biorad, Hercules, CA)). Each immunoblotting experiment was performed three times.

Statistics

Id2 and Id3 immunostaining scores in control and tumor tissue were compared using the Mann–Whitney two-tailed nonparametric *U*-test in SigmaPlot 12.0 (Systat Software, San Jose, CA). Id2, Id3 and Id4 immunostaining scores in patients less than 7 years of age were compared to patients who were 7 years and older using the same Mann–Whitney test. A two-tailed *t*-test was used to compare cells overexpressing either Id2 or Id3 to cells transfected with empty vector at various time points. Statistical significance was defined as $p<0.05$ for all tests. Data was plotted using SigmaPlot 12.0.

Results

Id Proteins are Differentially Expressed in Medulloblastoma Tumor Cells and Endothelia

Hematoxylin and eosin (H&E) stained sections of the 45 medulloblastoma cases on the tissue microarray showed that all except one was of classic histopathology as defined by the WHO; the lone exception belonged to the desmoplastic/nodular subtype (Fig. 1a–e) [1]. Id1 protein was not detected in tumor cells, but intense staining was observed in the endothelial nuclei of 78 % (35/45) of the medulloblastoma cases (Fig. 1f–j) (Table 3). Id2 protein immunostaining was positive in 89 % (40/45) of

Table 2 Antibodies for immunoblotting

Id protein antibodies	Dilution	Company	Product number
Id 1	1:200	Santa Cruz Biotechnology	sc-488
Id 2	1:200	Santa Cruz Biotechnology	sc-489
Id 3	1:200	Santa Cruz Biotechnology	sc-490
Id 4	1:200	Santa Cruz Biotechnology	sc-491
Cell cycle antibodies			
Cyclin D1	1:1000	Cell Signaling	2926
p27 Kip1	1:1000	Cell Signaling	2552
p15 INK4B	1:1000	Cell Signaling	4822
p16 INK4A	1:1000	Cell Signaling	4824
CDK6	1:1000	Cell Signaling	3136
Cyclin D3	1:1000	Cell Signaling	2936
p21 Waf/Cip1	1:1000	Cell Signaling	2946
CDK4	1:1000	Cell Signaling	2906
Retinoblastoma-underphosphorylated	1:250	BD Biosciences	554164
Retinoblastoma	1:250	BD Biosciences	554136
Cleaved PARP	1:500	Cell Signaling	5625
GAPDH	1:5000	Abcam	ab9484

Table 3 Demographics and clinical information of medulloblastoma tissue microarray patients and Id protein staining scores

	ID	Age	Sex	Id1	Id2 score	Id3 score	Id4 score
1	03-L6010	8 y 9 m	F	Endo +	1	4	0
2	04-L2068	7 y 9 m	F	Endo +	4	16	1
3	09-L6002	3 y 4 m	M	Endo +	0	6	0
4	11-L1203	2 y 1 m	M	Endo +	0	8	0
5	12-L4156	7 y 6 m	M	Endo +	2	12	0
6	02-L4074	15 y 7 m	M	Endo +	4	16	1
7	03-L4308	7 y 8 m	M	–	1	0	0
8	04-L4083	5 y 6 m	M	Endo +	3	16	0
9	11-L4024	5 y 1 m	M	Endo +	4	16	0
10	12-L6018	2 y 6 m	M	–	0	2	0
11	02-L1025	5 y 1 m	M	Endo +	9	16	1
12*	03-L1177	Unk	Unk	Endo +	0	8	1
13	04-L1024	3 y 6 m	F	Endo +	1	9	0
14	03-L010	9 y 4 m	M	Endo +	1	2	0
15	08-L025	4 y 7 m	M	–	4	0	0
16	10-L036	1 y 11 m	F	Endo +	6	16	0
17	10-L042	15 y 8 m	F	Endo +	4	9	1
18	05-L012	10 y 4 m	F	Endo +	1	9	0
19	05-L027	11 y 6 m	M	–	9	6	0
20	05-L055	7 y 3 m	M	Endo +	2	12	0
21	05-L098	9 y 1 m	M	Endo +	2	12	1
22	07-L048	7 y 3 m	M	–	4	12	0
23	08-L357	5 y 5 m	F	Endo +	0	8	0
24	09-L256	6 y 10 m	M	Endo +	1	16	1
25	03-L115	8 y 10 m	F	Endo +	2	16	0
26	06-L187	5 y 7 m	M	Endo +	9	16	0
27	06-L235	9 y 5 m	M	Endo +	4	16	0
28	07-L049	9 y 2 m	F	–	4	16	0
29	08-L352	4 y 2 m	F	Endo +	1	6	1
30	11-L014	16 y 9 m	F	Endo +	9	16	0
31	01-L141	8 m	M	Endo +	4	16	0
32	02-L061	10 y 7 m	F	Endo +	4	16	0
33	02-L113	1 y 8 m	M	–	9	16	0
34	04-L464	1 y 0 m	F	–	2	16	0
35	05-L600	2 y 7 m	F	–	1	6	0
36	10-L299	6 y 8 m	M	Endo +	4	12	0
37	10-L323	7 y 2 m	M	Endo +	1	16	0
38	11-L1254	1 y 11 m	F	Endo +	4	8	0
39	11-L461	5 y 4 m	M	–	2	6	0
40	12-L025	6 y 8 m	M	Endo +	2	12	0
41	01-L1137	3 y 8 m	M	Endo +	12	16	0
42	05-L2010	3 y 11 m	F	Endo +	12	16	0

Table 3 (continued)

	ID	Age	Sex	Id1	Id2 score	Id3 score	Id4 score
43	07-L2266	9 y 10 m	M	Endo +	12	16	0
44	10-L1212	15 y 7 m	M	Endo +	4	16	0
45	10-L2076	10 y 7 m	F	Endo +	6	12	0

* desmoplastic/ nodular subtype; *Endo* endothelial nuclear staining; *F* female; *ID* specimen identification number; *M* male; *m* month; *y* year; *Unk* unknown

cases, but the staining was variable in medulloblastoma tumor nuclei (Fig. 1k–o) and ranged from tumors that had no

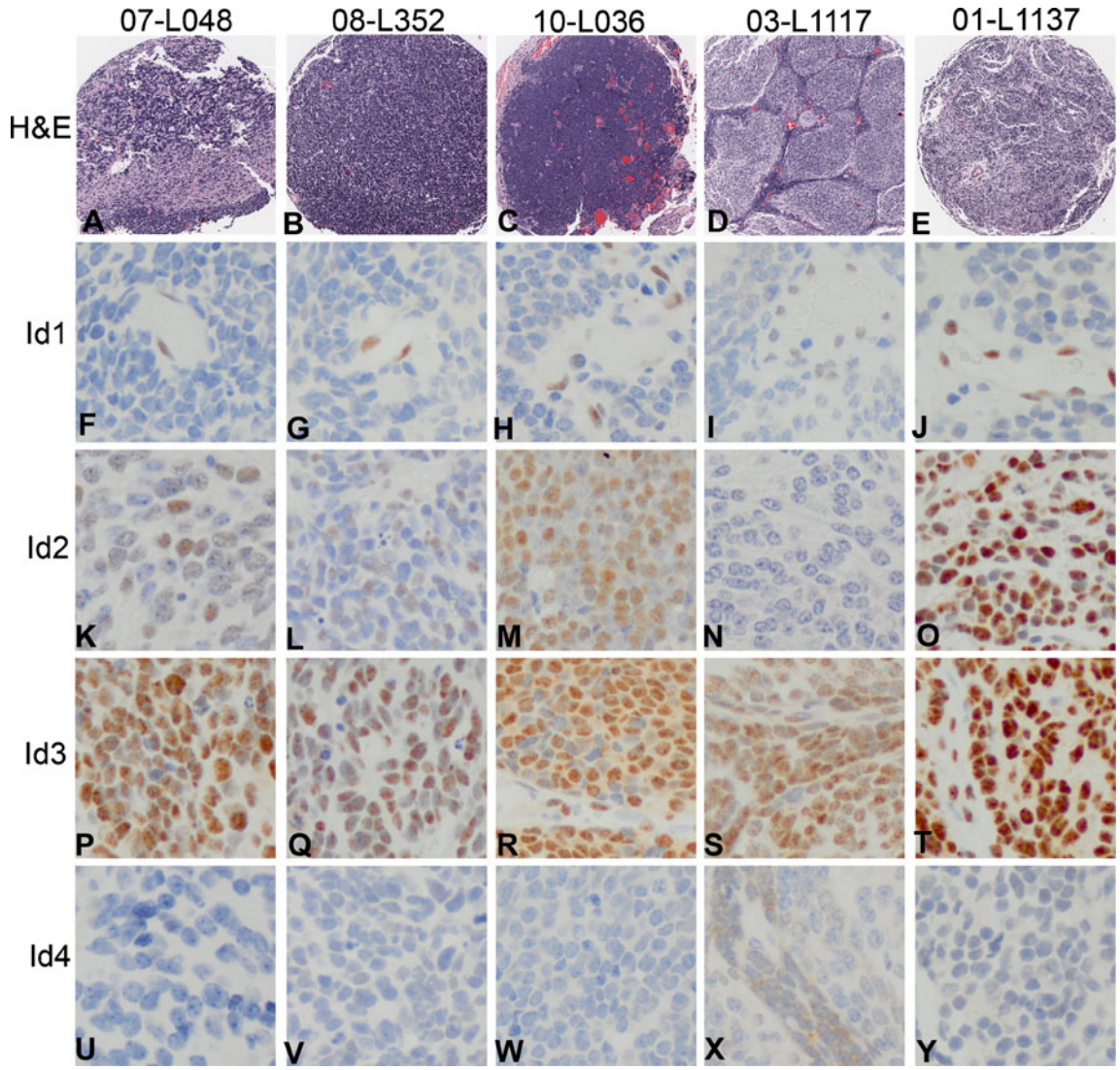

Fig. 1 Id proteins are differentially expressed in medulloblastoma tumor cells and endothelial cells. Hematoxylin and eosin (H&E) stained sections of five medulloblastoma tumors on the tissue microarray (**a**–**e**). All of the tumors on the tissue microarray had classic histology except **d**, which was desmoplastic/nodular subtype. Id1 protein expression is not expressed in tumor cells and is limited to endothelial cell nuclei of the tumor vasculature (**f**–**j**). Id2 is variably expressed in medulloblastoma tumor cells (**k**–**o**). Id3 is highly expressed in medulloblastoma tumor cells and endothelial nuclei (**p**–**t**). Id4 (**u**–**y**) is not expressed in medulloblastoma aside from some weak expression in the internodular zone of the desmoplastic/nodular tumor (**x**). Original magnification: 100× in **a**–**e** (bar is 100 μm) and 400× in **f**–**y** (bar is 25 μm)

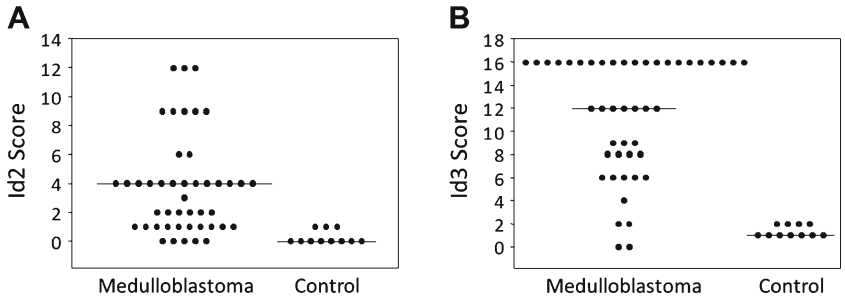

Fig. 2 Dot plot of Id2 and Id3 staining scores with a line designating median staining scores. **a** The median Id2 staining score in medulloblastoma (N=45) is 4, while the median staining score in normal cerebellar cortex (N=11) is 0 (p<0.001, Mann–Whitney U-test). **b** The median Id3 staining score in medulloblastoma is 12 (N=45), which is significantly higher than the median score in normal cerebellar cortex (N=11) of 1 (p<0.001, Mann–Whitney U-test)

immunoreactivity (Fig. 1n) to others with intense immunoreactivity (Fig. 1o) (Table 3). Id3 protein expression was more uniformly increased in medulloblastoma tumor nuclei and in tumor endothelial nuclei (Fig. 1p–t) with 98 % (44/45) of medulloblastomas demonstrating immunoreactivity (Table 3). Id4 is not expressed to a significant degree in medulloblastoma (Fig. 1u–y) and only 18 % of cases (8/45) showed weak immunoreactivity (Table 3). The internodular zone of the only desmoplastic/nodular medulloblastoma on the tissue microarray was weakly Id4 immunoreactive, but the nodular areas were negative (Fig. 1x).

Id2 protein expression is elevated in medulloblastoma (N=45) with a median staining score of 4, while the median staining score in normal cerebellar cortex (N=11) is 0 (p<0.001, Mann–Whitney U-test) (Fig. 2a). Id3 staining was significantly elevated in medulloblastoma tumor cells with a median score of 12 (N=45), compared to normal cerebellar cortex (N=11), with a median score of 1 (p<0.001, Mann–Whitney U-test) (Fig. 3b). Id proteins are developmentally regulated so Id2, Id3 and Id4 immunostaining scores were compared between patients who are less than 7 years old and those who were 7 years of age or older. Twenty-four patients

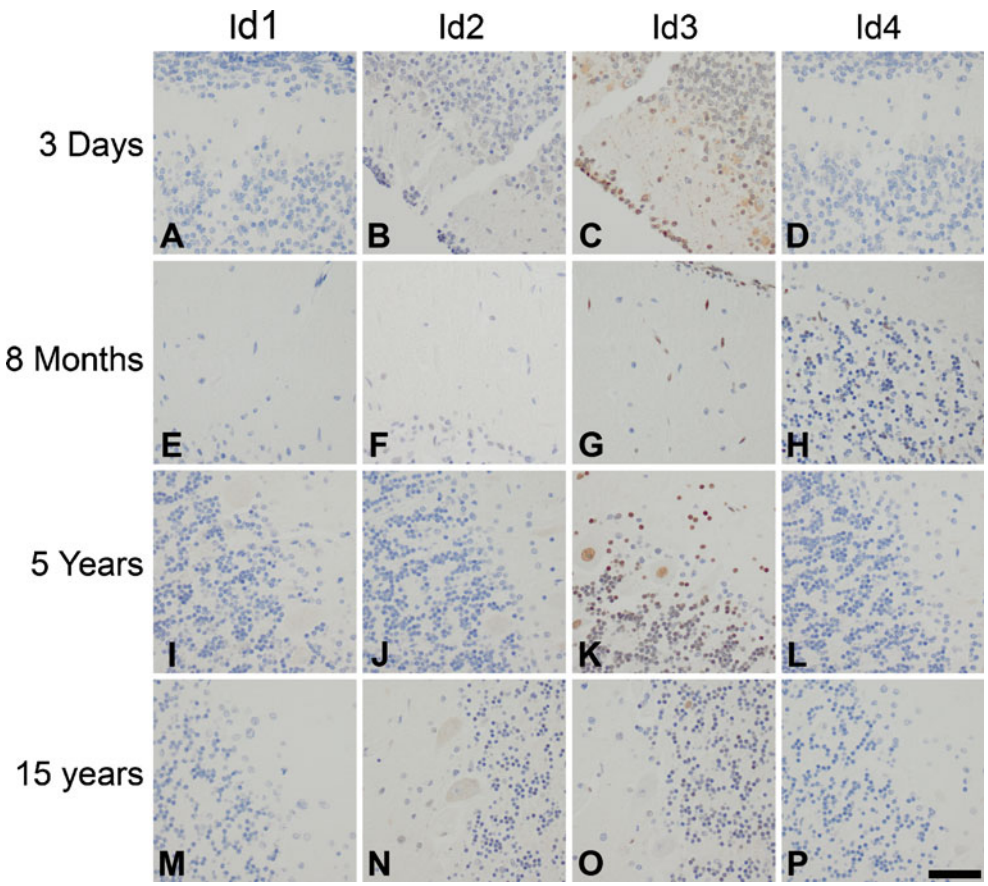

Fig. 3 Id proteins are differentially expressed during the development of the normal human cerebellar cortex. Normal human cerebella from a wide range of ages (3 days, 8 months, 5 years and 15 years) were used as controls. Id3 was the only Id protein detected at 3 days (**a**–**d**) and at 8 months of age (**e**–**h**). Id3 expression appeared decreased at 8 months relative to 3 days of age. At 5 days of age (**i**–**l**) scattered internal granule cells and Purkinje cells expressed Id3 (**k**). No Id protein expression was detected in cerebellar cortex at 15 years of age (**m**–**p**). Original magnification is 400×; bar is 25 μm

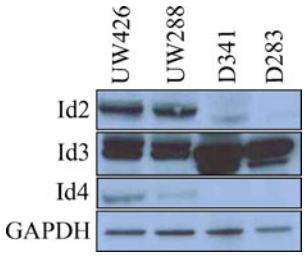

Fig. 4 Id proteins are expressed in medulloblastoma cell lines. Id2 expression was noted in UW426 and UW288 cells, with minimal expression in D341 cells and no expression in D283 cells. All four cell lines expressed Id3 at high levels. Minimal Id4 protein expression was noted in UW426 and UW288 lines, but not in D341 or D283. Id1 was not detected in any cell line (data not shown)

were less than 7 years old and 20 were 7 years or older. One patient's age was unknown. There was no significant difference in Id2 (<7 years: 3.9±3.7; ≥7 years: 3.9±3.1; p=0.718), Id3 (<7 years: 11.5±5.2; ≥7 years: 11.7±5.2; p=0.930) or Id4 (<7 years: 0.1±0.3, ≥7 years: 0.2±0.4; p=0.515) immunostaining scores between the two age groups.

Id protein expression is minimal in normal cerebellar cortex (Fig. 3a–p). Id1, Id2 and Id4 are not expressed in normal cerebellar cortex at any of the studied time points. Id3 protein is expressed in external granule neurons, Purkinje cells and endothelial cells at 3 days (Fig. 3c) and 8 months (Fig. 3g). Purkinje cell and granule neuron Id3 expression remains strong at 5 years (Fig. 3k), but at 15 years of age Id3 protein expression is minimal and is only noted in rare, scattered granule neurons (Fig. 3o).

Taken together these data show that Id2 and Id3 proteins are differentially expressed in medulloblastoma tumor cells relative to controls. Id3 expression shows the most significant up regulation in medulloblastoma and its expression in infant cerebellar cortex suggests that it could have an important role in development. Id1 is not expressed in endothelial cells from normal cerebellar cortex, but the majority of tumors show substantial Id1 expression in tumor endothelial nuclei. Id3 is also expressed in tumor and normal endothelial nuclei.

Id Protein Expression is Altered in Human Medulloblastoma Cell Lines

Id protein expression was studied in UW288, UW426, D341 and D383 medulloblastoma cell lines (Fig. 4). Id1 protein was not detected in any of the cell lines studied (data not shown). Abundant Id2 protein expression was detected in UW426 and UW288 and these cell lines also express Id4. Id3 was abundantly expressed in all four of the tested medulloblastoma cell lines. These data show that medulloblastoma cell lines model the Id protein expression profile of human tumors. We used UW426 and UW288 cell lines in subsequent experiments as the Id protein expression profile in these cell lines most accurately modeled Id protein expression in human medulloblastoma.

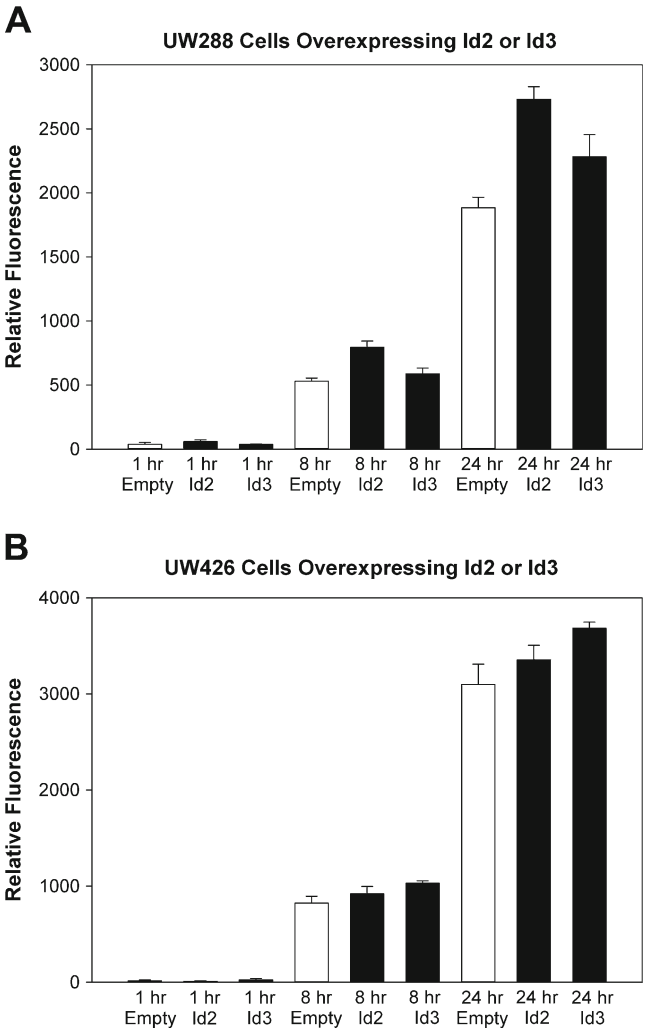

Fig. 5 Id2 and Id3 overexpression drives UW426 and UW288 cell proliferation. Cell proliferation was assessed using the AlamarBlue cell proliferation assay and by quantitating relative fluorescence at 1, 8 and 24 h after plating. UW288 cells overexpressing Id2 showed 33 % more relative fluorescence than control cells expressing empty vector at 8 h (p<0.001), and 31 % more relative fluorescence than empty vector controls at 24 h (p<0.001) (**a**). UW288 cells overexpressing Id3 showed 10 % more relative fluorescence (p<0.01) than controls at 8 h and 17 % more relative fluorescence (p<0.001) at 24 h than empty vector controls (**a**). UW426 cells overexpressing Id2 showed a 10 % increase in relative fluorescence (p=0.11) at 8 h and an 8 % increase in relative fluorescence (p=0.04) relative to empty vector controls at 24 h (**b**). UW426 cells overexpressing Id3 showed a 20 % increase in relative fluorescence (p<0.001) at 8 h and a 16 % increase in relative fluorescence (p=0.002) relative to empty vector controls at 24 h (**b**)

Id2 and Id3 Overexpression Enhances Cell Proliferation in Medulloblastoma Cell Lines

Id protein expression is increased in proliferating, undifferentiated cells. To determine the effects of Id2 and Id3 on

medulloblastoma tumor cells the UW426 and UW288 cell lines were stably transfected to drive the expression of Id2 and Id3. Western data showed that the Id2 and Id3 protein expression levels were doubled relative to empty vector controls (data not shown). AlamarBlue assay was performed to quantitate cell proliferation. Fluorescence was determined at 1 h, 8 h and 24 h after plating. No significant difference was noted in relative fluorescence between UW288 cells transfected with empty vector or cells overexpressing Id2 (p=0.79) or Id3 (p=0.4) at 1 h. UW288 cells overexpressing Id2 showed 33 % more relative fluorescence (p<0.001) than control cells expressing empty vector at 8 h, while UW288 cells overexpressing Id3 showed 10 % more relative fluorescence (p<0.01) than controls at this time point (Fig. 5a). At 24 h Id2 overexpressing UW288 cells showed 31 % more relative fluorescence (p<0.001) while Id3 overexpressors showed 17 % more relative fluorescence (p<0.001) than controls (Fig. 5a). No significant difference was noted in relative fluorescence between UW426 cells transfected with empty vector or cells overexpressing Id2 (p=0.47) or Id3 (p=0.34) at 1 h. Id2 overexpressing UW426 cells showed 10 % more relative fluorescence (p=0.11) while Id3 overexpressors showed a 20 % increase in relative fluorescence (p<0.001) when compared to empty vector controls at 8 h (Fig. 5b). At 24 h UW426 cells overexpressing Id2 showed an 8 % increase in relative fluorescence (p=0.04) while Id3 overexpressing UW426 cells showed a 16 % increase in relative fluorescence (p=0.002) compared to empty vector controls. These increases were not due to differences in cell death as immunoblotting using antibodies specific to cleaved-PARP, a marker of apoptosis, showed no difference amongst Id overexpressing cells or controls in either cell line (data not shown). Taken together, these data show that Id2 and Id3 proteins potentiate cell proliferation in the UW288 and UW426 medulloblastoma cell lines.

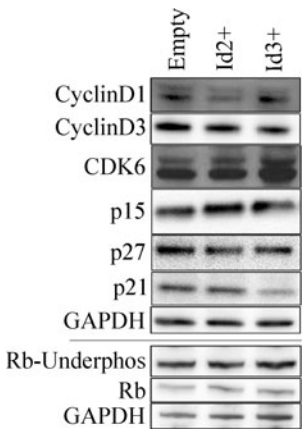

Fig. 6 Id2 and Id3 protein overexpression perturb cell cycle protein regulation in UW288 cells. Cyclin D1 was decreased 36 % in Id2 over expressing cells, but no change was noted in Cyclin D3 in medulloblastoma cells overexpressing Id2 or Id3. There was no change in CDK6 expression. No change was noted in p15 expression and p16 was not detectable (data not shown). Id2 or Id3 over expression decreased p27 expression 48 % and 35 %, respectively. Over expression of Id2 or Id3 decreased p21 expression 16 % and 60 %, respectively. p57 was not detectable (data not shown). Id2 and Id3 over expression increased phosphorylated retinoblastoma by 39 % and 46 %, respectively

Id2 and Id3 Overexpression Deregulates the Expression of Critical Cell Cycle Regulatory Proteins in Medulloblastoma Cells

Cell cycle progression is tightly regulated and is dependent on the proper coordination of cyclin dependent kinases (CDK). CyclinD/CDK4/6 complex activity hyperphosphorylates retinoblastoma, initiates S-phase, and commits a cell to proliferate by releasing HLH transcription factors. Cip/-Kip protein family members such as p21, p27 and p57 act as CDK inhibitors and block the transition to S-phase. To study the effects of Id2 and Id3 on cell cycle regulation we performed immunoblotting of UW288 cells overexpressing Id2 or Id3 relative to empty vector controls. Cyclin D1 was decreased 36 % in Id2 over expressing cells, but no change was noted in Id3 overexpressing cells (Fig. 6). Cyclin D3 and CDK6 expression was not altered by Id2 or Id3 overexpression in medulloblastoma cells (Fig. 6). No change in p15 was noted and p16 was not detectable in medulloblastoma cells (data not shown). Independent overexpression of Id2 or Id3 decreased p27 expression by 48 % and 35 %, respectively (Fig. 6). Overexpression of Id2 decreased p21 expression 16 %, while Id3 overexpression decreased p21 expression 60 % (Fig. 6), while p57 was not detectable (data not shown). Id2 and Id3 over expression in UW288 cells increased phosphorylated retinoblastoma by 39 % and 46 %, respectively (Fig. 6). These data show that the up regulation of Id2 and Id3 expression drives cell proliferation by perturbing the regulation of cell cycle proteins resulting in increased retinoblastoma phosphorylation and decreased expression of Cip/Kip family proteins.

Discussion

Most studies of Id protein expression in the developing mammalian nervous system are based on rodent tissues [15, 16], with relatively few studies performed in human tissues [17]. In mice, proliferating, relatively undifferentiated neuroepithelial cells show abundant Id1 and Id3 protein expression and then levels decline and become undetectable in the brains of adult mice [6]. Id1 and Id3 are co-expressed during murine neurogenesis [4, 18, 19] and angiogenesis [4, 5]. Id2 is also expressed in the early phases of central nervous system development; however, in contrast to Id1 and Id3, Id2 expression is retained postnatally in mice and

persists in maturing neurons of the cerebral cortex and Purkinje cells of the cerebellar cortex [5, 6]. Our data show that Id3 is expressed at substantial levels, while Id2 is minimally expressed in the early postnatal human cerebellar cortex, specifically in external and internal granule cells and Purkinje cells up to 5 years of age. It is possible that Id3 could regulate neuronal differentiation or survival and the role of Id3 in human cerebellar development merits further study.

Increased Id protein expression has been reported in many different cancers and tends to be associated with high-grade, often aggressive tumors (reviewed by Fong et al. [7]), which is in keeping with our findings of increased Id2 and Id3 expression in medulloblastoma tumor cells. Information regarding Id protein expression in brain tumors is limited, but it has been studied in gliomas where Id1, Id2 and Id3 expression are up regulated in high grade tumors and correlate with tumor proliferation rate [20]. Id proteins are important in tumor angiogenesis and endothelial expression of Id1, Id2 and Id3 are up regulated in gliomas, but not detectable in native vessels [20]. In contrast, we detected Id1 and Id3, but not Id2 in endothelial cells of medulloblastoma tumor vessels. Other brain tumor types should be studied to determine if up regulating Id protein expression is a general mechanism in brain tumor biology.

Our data shows that forced Id2 and Id3 overexpression enhanced medulloblastoma cell proliferation, which was accompanied by decreased expression of two key cyclin dependent kinase inhibitors: p21 and p27. Id2 and Id3 are known to modulate cell cycle progression by down regulating p21 and p27 [21, 22]. Id3 in particular, plays a critical role in cell cycle progression and survival of neural crest progenitors by regulating p27 and the Id1 −/− Id3 +/− knockout mouse shows a reduction in proliferating neuroepithelial cells that is accompanied by an up regulation of p27 [5]. Cerebellar granule cell proliferation is tightly controlled by p27, which suppresses the development of medulloblastoma in *Patched 1* heterozygous mice [23, 24]. Future experiments will further examine the role of Id3 and cyclin dependent kinase inhibitors in medulloblastoma biology.

Studies have shown that the expression profile of HLH transcription factors and Id proteins is important to the biology of embryonal brain tumors like medulloblastoma [25]. However, little is known regarding the role of Id proteins in human cerebellar granule precursor cells, which are believed to give rise to a subset of medulloblastomas characterized by altered sonic hedgehog (SHH) signaling [26]. SHH induces Id2 and N-myc expression, which drives the proliferation of cerebellar granule cells in vitro [27]. Furthermore, in granule cells differentiation is coupled to Id2 degradation, which initiates cell cycle exit and axonal growth [28]. Because the modulation of Id protein levels is critical to granule precursor cell proliferation and differentiation, it is possible that perturbations in postnatal Id protein expression in these cells have a key role in medulloblastoma pathogenesis.

Forced Id2 and Id3 overexpression in medulloblastoma cell lines increased levels of phosphorylated retinoblastoma protein. Id2 directly binds retinoblastoma, preventing formation of the retinoblastoma/E2F complex and allowing cell cycle progression from G1 to S phase [29]. However, the biological significance of Id2 and retinoblastoma maybe even more complex than this as there also appears to be a genetic interaction between retinoblastoma and Id2, since an *ID2* knockout can partially rescue the lethal phenotype of the retinoblastoma knock out mouse, indicating that retinoblastoma may be restraining Id2 activity [29, 30]. Some have speculated about the possibility of a retinoblastoma-Id2-p16 pathway, where retinoblastoma loss, as is common in many cancers, leads to an increase in Id2, which in turn sequesters more transcription factors, decreasing levels of p16, a mediator of cellular senescence [8, 31]. We were unable to detect p16 in untransfected control and Id overexpressing medulloblastoma cells, so this mechanism may not occur in medulloblastoma. How Id2 contributes to medulloblastoma tumor cell proliferation will be the subject of future studies.

Clinical information was only available from 16 medulloblastoma patients whose tumors where represented on the tissue microarray so no survival analysis was possible and we were unable to correlate Id protein expression with patient outcome. This is a limitation of the study; however, since Id2, and especially Id3 expression levels, were consistently elevated in the majority of the tumors we studied it seems unlikely that a differential pattern of Id protein expression could be correlated with clinical outcome.

Taken together our findings show that Id proteins are differentially expressed in medulloblastoma tumor cells and endothelia, and that Id2 and Id3 overexpression enhances cell proliferation in the former. Future work can address the suitability of exploiting Id proteins as a therapeutic target in medulloblastoma. Id proteins are attractive therapeutic targets because they are involved in multiple aspects of tumor biology, allowing many facets of the malignant phenotype to be conveniently targeted using a single approach. Therapies targeting Id proteins could be potent, as supported by studies in preclinical models suggesting that even a partial reduction of Id levels can show therapeutic benefit [3, 7, 8, 13]. Furthermore, Id proteins are highly expressed in tumor cells and tumor blood vessels, but minimally expressed in normal, mature tissues; indicating that a therapeutic approach directed against Id proteins would be expected to have limited toxicity in normal tissues. This property of Id proteins would be an asset in our search for ways to refine therapy, with the goal of ameliorating the life-

long complications currently experienced by survivors of medulloblastoma.

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