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Notch- and vitamin D signaling in 1,25(OH)$_2$D$_3$-resistant glioblastoma multiforme (GBM) cell lines☆

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ABSTRACT

Recently, an important role of Notch activation for Ras-induced transformation of glial cells and for glioma growth and survival has been demonstrated. It was concluded that activation of Notch-signaling may represent a new target for glioblastoma multiforme (GBM) therapy. We now analyzed five GBM cell lines (Tx3095, Tx3868, U87, U118, U373) for key components of Notch-signaling pathways (Notch-1, Notch-2, Notch-3, Notch-4, Delta-like 1, Delta-like 3, Delta-like 4, Jagged-1, Jagged-2) using conventional RT-PCR. We found that some components (Notch-1, Notch-2, Notch-4, Jagged-1) were consistently expressed in all cell lines analyzed while, in contrast, other key components of Notch-signaling were differentially expressed. Notch-3 was expressed in three out of five cell lines (in U87, U118 and U373), but was missing in Tx3095 and Tx3868 cells. Jagged-2 was expressed in U87, U373 and Tx3868, but not in U118 or Tx3095 cells. Delta-like 1 and Delta-like 3 were not detected in Tx3905 cells, but in all other cell lines. RNA for Delta-like 4 was only found in U373 and Tx3868 GBM cell lines. Treating GBM cell lines with 1,25(OH)$_2$D$_3$ (10^{-6}, 10^{-8}, and 10^{-10} M), the biologically active form of vitamin D, did not result in significant dose- or time-dependent antiproliferative effects, indicating that GBM cell lines are resistant against the antiproliferative activity of 1,25(OH)$_2$D$_3$. *In vitro* treatment of GBM cells with 1,25(OH)$_2$D$_3$ did not result in a modulation of the expression of key components of the Notch-signaling pathway. Treatment with HDAC-inhibitor TSA or DNA-methyltransferase inhibitor 5-aza exerted dose- and time-dependent antiproliferative effects on GBM cell lines. We asked the question whether the resistance against 1,25(OH)$_2$D$_3$ could be restored by co-treatment with TSA or 5-aza. However, combination therapy with 1,25(OH)$_2$D$_3$ and TSA or 5-aza did not result in enhanced antiproliferative effects as compared to treatment with TSA or 5-aza alone. In contrast, antiproliferative effects of TSA and 5-aza were partially antagonized by concomitant treatment with 1,25(OH)$_2$D$_3$, indicating a protective effect of 1,25(OH)$_2$D$_3$ against the antiproliferative effects of TSA and 5-aza in GBM cell lines. In conclusion, our findings point at a differential expression of key components of Notch-signaling in GBM cell lines that may be of importance for the growth characteristics of GBM. Our findings indicate that GBM cell lines are resistant against the antiproliferative effects of 1,25(OH)$_2$D$_3$, and that this resistance may not be overcome by modulation of epigenetic silencing. Our findings do not support the hypothesis that modulation of Notch-signaling pathways by 1,25(OH)$_2$D$_3$ may regulate growth of GBM cell lines.

1. Introduction

Recently, an important role of Notch activation for Ras-induced transformation of glial cells and for glioma growth and survival has been demonstrated [1,2]. Additionally, these findings indicate that activation of Notch-signaling may represent a new target for glioblastoma multiforme (GBM) therapy. Increasing evidence indicates that the vitamin D endocrine system is implicated in pathogenesis and progression of various malignancies. It is well known that 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$), the biologically most active natural vitamin D metabolite, acts *via* binding to its corresponding intranuclear receptor (VDR), present

Abbreviations: 5-aza, 5-azacytidine; Dll, Delta-like; 1,25(OH)$_2$D$_3$, 1,25-dihydroxyvitamin D$_3$; DNMTI, DNA-methyltransferase inhibitor; GBM, glioblastoma multiforme; HDACI, histone deacetylase inhibitor; jag, Jagged; PCR, polymerase chain reaction; TSA, trichostatin A.

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in target tissue cells [3]. In various normal and malignant cell types the effects of $1,25(OH)_2D_3$ include the regulation of cell growth and differentiation [4–6]. Modulation of Notch and/or vitamin D-signaling pathways may represent a potential novel strategy to prevent carcinogenesis of GBM and to treat GBM. It was the aim of this study to analyze the expression of key components of Notch-signaling pathway, and their modulation by treatment with $1,25(OH)_2D_3$, in several GBM cell lines *in vitro*.

2. Materials and methods

2.1. Cell lines

Several glioma cell lines (Tx3868, Tx3095, U87MG, U118) were maintained in DMEM (10% FBS, glutamine, and Pen/Strep), the glioma cell line U373MG was treated with RPMI (10% FBS, glutamine, and Pen/Strep), as reported preciously [7]. $1,25(OH)_2D_3$ and trichostatin A (TSA, Sigma–Aldrich, Taufkirchen, Germany) were dissolved in ethanol as a stock solution and stored in the dark at −20 °C, 5-azacytidine (5-aza, Sigma–Aldrich) was dissolved in PBS. For analysis of cell proliferation, cells were grown in 96-well plates (1×10^3 cells per well). Media containing varying concentrations of $1,25(OH)_2D_3$, TSA or 5-aza and their combinations were added to a final volume of 100 μl per well and plates were incubated for 24, 48, and 72 h, with re-dosing on a daily basis.

2.2. Proliferation assay

Cell proliferation was investigated in 96-well plates using a colorimetric immunoassay, based on the measurement of BrdU incorporation during DNA synthesis (BrdU ELISA kit Roche Diagnostics, Mannheim, Germany). In brief, BrdU (10 mM 5-bromo-2′-deoxyuridine) was added to the cells after 24 h of each daily treatment before cells were fixed, DNA was denaturated and a peroxidase labelled anti-BrdU-antibody was added. Immune complexes were detected by the subsequent substrate (tetramethyl-benzidine) reaction, which is quantified by measuring the absorbance values that directly correlate to the amount of DNA synthesis and therefore the number of proliferating cells.

2.3. Western blotting

For protein isolation, cells from 6-well plates were lysed in 50 μl of a lysis buffer heated to 100 °C and containing 50 mM Tris–HCl (pH 6.8), 100 mM DTT, 2% SDS and 20% glycerol. Samples containing 5 μg of total cellular protein were subjected to 8% SDS-PAGE and transferred to a PVDF membrane (Immobilon-P, Millipore, Bedford, USA). Signals were detected upon overnight incubation of the membranes with specific primary antibodies directed against target proteins followed by a final incubation with a peroxidase-conjugated secondary antibody and Renaissance Enhanced Luminol Reagents (NEN, Boston, USA), performed as specified by the supplier.

2.4. Polymerase chain reaction (PCR)

RNA from cell lines was prepared using the RNeasy kit (Qiagen, Hilden, Germany). cDNA was synthesized from 1 μg RNA per sample using super script II (Invitrogen, Karlsruhe, Germany).

2.5. Flow cytometry (fluorescence-activated cell sorting, FACS)

The cells (5×10^5) were seeded in six-well plates. Cells were collected and detected by flow cytometry according to the instructions of the manufacturer (Becton-Dickinson, San Jose, CA, USA).

3. Results

3.1. RNA expression of key components of the Notch-signaling pathway in GBM cell lines

We found by RT-PCR that some components (Notch-1, Notch-2, Notch-4, and Jagged-1) were consistently expressed in all cell lines analyzed (Fig. 1). In contrast, other components of Notch-signaling were differentially expressed in these five GBM cell lines (Fig. 1). Notch-3 was expressed in three out of five cell lines (U87MG, U118, and U373) but was missing in Tx3095 and Tx3868 cells. Jagged-2 was expressed in U87, U373 and Tx3868 but not in U118 or Tx3095 cells. Dll-1 was not detected in Tx3095 cells, but was found in all other cell lines. Dll-3 was weakly expressed in Saos-2 sarcoma cells (control) but not detected in the five GBM cell lines. RNA for

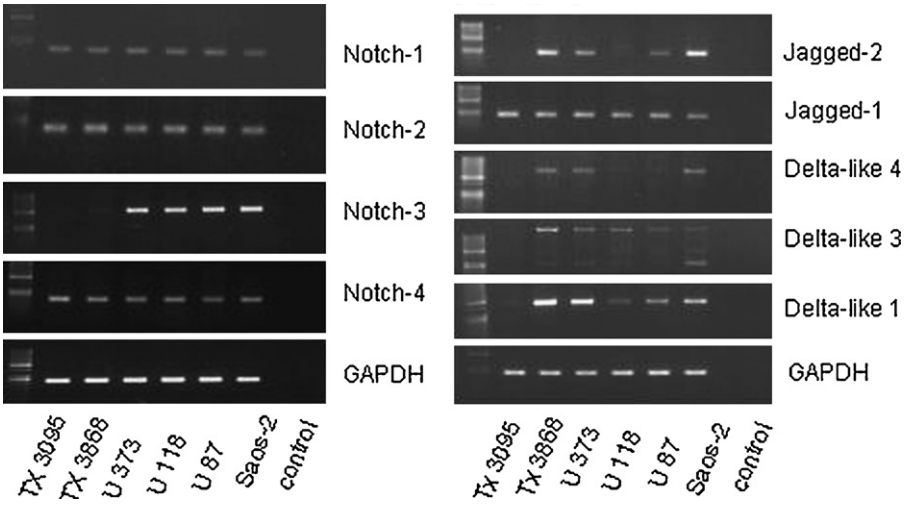

Fig. 1. We found by RT-PCR that some components (Notch-1, Notch-2, Notch-4, and Jagged-1) were consistently expressed in all cell lines analyzed. In contrast, other components of Notch signaling were differentially expressed in these five GBM cell lines. Notch-3 was expressed in three out of five cell lines (U87MG, U118, and U373) but was missing in Tx3095 and Tx3868 cells. Jagged-2 was expressed in U87, U373 and Tx3868 but not in U118 or Tx3095 cells. Dll-1 was not detected in Tx3095 cells, but was found in all other cell lines. Dll-3 was weakly expressed in Saos-2 sarcoma cells (control) but not detected in the five GBM cell lines. RNA for Dll-4 was only found in U373 and Tx3868 GBM cell lines. Note that Saos-2 sarcoma cells express all components of Notch signaling analyzed.

Dll-4 was only found in U373 and Tx3868 GBM cell lines. Note that Saos-2 sarcoma cells express all components of Notch-signaling analyzed.

3.2. Protein expression of key components of the Notch-signaling pathway in GBM cell lines

Western analysis revealed that Notch-3 and Notch-4 are expressed in all GBM cell lines analyzed, while Jagged-1 and Jagged-2 are expressed only in some cell lines (Fig. 2). Note that GBM cell lines express either Jagged-1 or Jagged-2 (Fig. 2). Spontaneously immortalized HaCaT keratinocytes, cutaneous squamous carcinoma cells (SCL-1), and sarcoma cells (Saos-2) were used as controls. FACS analysis confirmed the Jagged-2 and Notch-4 protein expression data obtained by Western analysis (Fig. 3).

3.3. Treatment of GBM cell lines with 1,25(OH)$_2$D$_3$ does not modulate protein expression of Notch-2, Notch-4, or their corresponding ligand Jagged-1

Western analysis revealed that treatment with 1,25(OH)$_2$D$_3$ does not modulate protein expression of Notch-2, Notch-4, or their corresponding ligand Jagged-1 in GBM cell lines U87 and Tx3095 *in vitro* (Fig. 4).

3.4. Treatment of GBM cell lines with 1,25(OH)$_2$D$_3$, TSA, 5-aza and their combination

Treating GBM cell lines with 1,25(OH)$_2$D$_3$ (10^{-6}, 10^{-8}, and 10^{-10} M) every 24 h did not result in significant dose- or time-dependent antiproliferative effects, indicating that GBM cell lines are resistant against the antiproliferative activity of 1,25(OH)$_2$D$_3$ (Fig. 5). Treatment with HDAC-inhibitor TSA (Fig. 5A) or DNA-methyltransferase inhibitor 5-aza (Fig. 5B) exerted dose- and time-dependent antiproliferative effects on GBM cell lines. We asked the question whether the resistance against 1,25(OH)$_2$D$_3$ could be restored by co-treatment with TSA or 5-aza. However, combination therapy with 1,25(OH)$_2$D$_3$ and TSA (Fig. 5A) or 5-aza (Fig. 5B) did not result in stronger antiproliferative effects

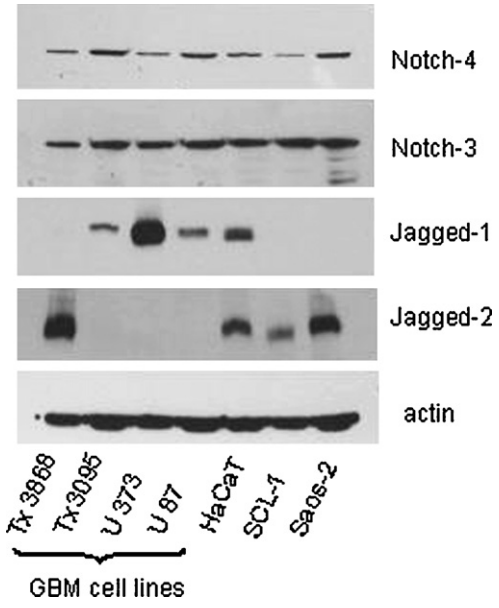

Fig. 2. Western blot from different GBM cell lines: Notch-3 and Notch-4 are expressed in all GBM cell lines analyzed, while Jagged-1 and Jagged-2 are expressed only in some cell lines. Note that GBM cell lines express either Jagged-1 or Jagged-2. Spontaneously immortalized HaCaT keratinocytes, cutaneous squamous carcinoma cells (SCL-1), and sarcoma cells (Saos-2) were used as controls.

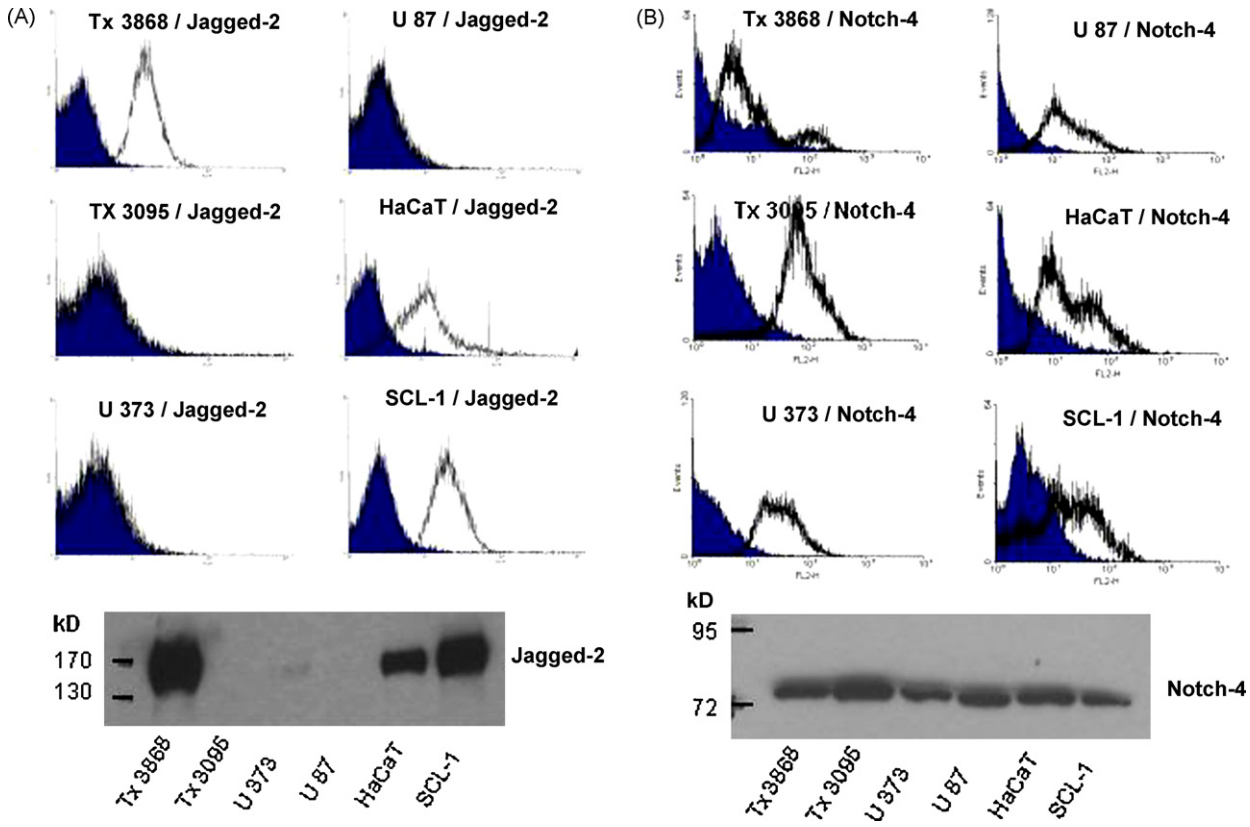

Fig. 3. The Jagged-2 (A) and Notch-4 (B) protein expression data obtained by Western blot and FACS analysis are comparable.

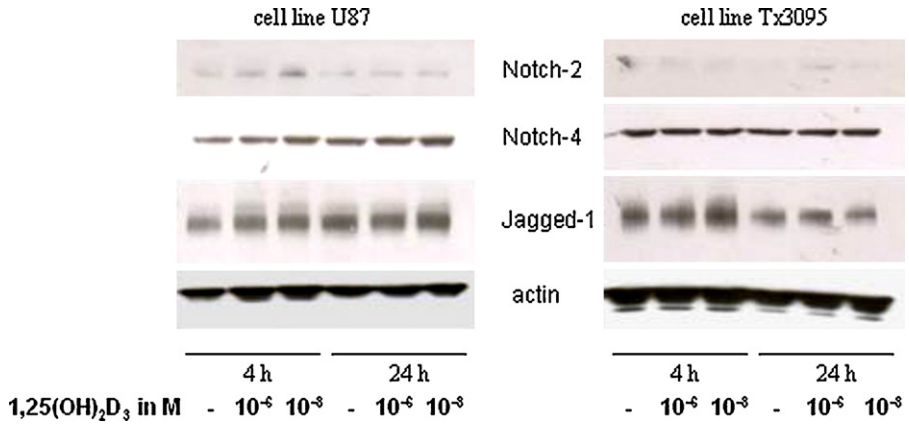

Fig. 4. Western blot from GBM cell lines U87 and Tx3095: Note that treatment with $1,25(OH)_2D_3$ does not modulate protein expression of Notch-2, Notch-4, or their corresponding ligand Jagged-1.

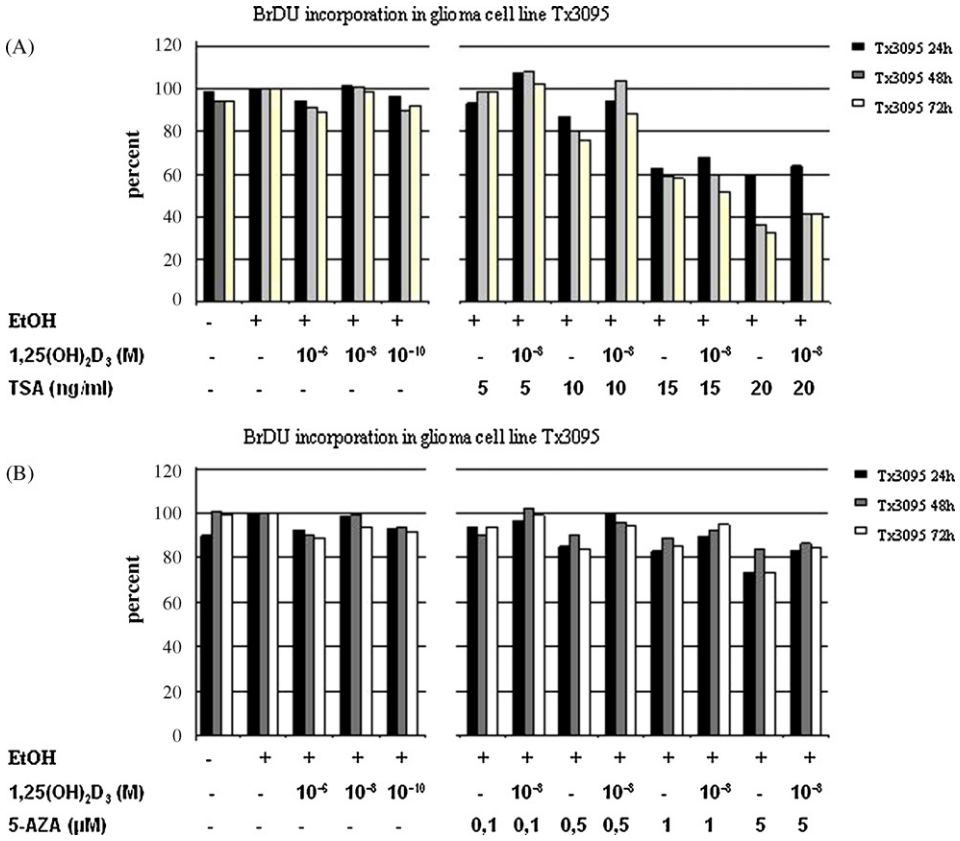

Fig. 5. Treating GBM cell lines with $1,25(OH)_2D_3$ (10^{-6}, 10^{-8}, and 10^{-10} M) every 24 h for 72 h does not result in significant dose- or time-dependent antiproliferative effects, indicating that GBM cell lines are resistant against the antiproliferative activity of $1,25(OH)_2D_3$ (A and B). Treatment with HDAC-inhibitor TSA (A) or DNA-methyltransferase inhibitor 5-aza (B) exerts dose- and time-dependent antiproliferative effects on GBM cell lines. We asked the question whether the resistance against $1,25(OH)_2D_3$ could be restored by co-treatment with TSA or 5-aza. However, combination therapy with $1,25(OH)_2D_3$ and TSA (A) or 5-aza (B) does not result in stronger antiproliferative effects as compared to treatment with $1,25(OH)_2D_3$ alone. In contrast, antiproliferative effects of TSA (A) and 5-aza (B) are partially antagonized by concomitant treatment with $1,25(OH)_2D_3$, indicating a protective effect of $1,25(OH)_2D_3$ against the antiproliferative effects of TSA and 5-aza in GBM cell lines.

as compared to treatment with TSA or 5-aza alone. In contrast, antiproliferative effects of TSA (Fig. 5A) and 5-aza (Fig. 5B) were partially antagonized by concomitant treatment with $1,25(OH)_2D_3$, indicating a protective effect of $1,25(OH)_2D_3$ against the antiproliferative effects of TSA and 5-aza in GBM cell lines.

4. Discussion and conclusions

Our findings point to a differential expression of key components of Notch-signaling pathways in GBM cell lines, that may be important for the growth characteristics of GBM and may have future therapeutic implications. Most interestingly, GBM cell lines analyzed in this study express either Jagged-1 or Jagged-2. There is increasing evidence that tumor stem cells are of high importance for pathogenesis and progression of GBM. Recently, it was shown that active Jagged-1 is required for the maintenance of multipotent adult-type neutral stem cells *in vitro* and mitotic cells in the postnatal subventricular zone [8]. Moreover, it has been speculated that Jagged-1 may, through activation of Notch-1, maintain progenitor cells of the CNS, and that reduced Jagged-1/Notch-1 signaling

may result in a reduction of proliferating cells in the subventricular zone and in the rostral migratory stream [8]. It can be speculated whether the differential expression of Jagged-1 or Jagged-2 reported in this study may be of importance for progression of GBM, or may represent a prognostic marker or a target for future therapeutic strategies. The limited success of available treatments underlines the needs to develop new therapeutic and preventive approaches for GBM. The vitamin D endocrine system has been implicated in the pathogenesis and progression of various malignancies [4–6]. Moreover, $1,25(OH)_2D_3$, the biologically most active natural vitamin D metabolite that acts *via* binding to its corresponding intranuclear receptor (VDR), present in target tissue cells [3] has been considered as a potential anticancer agent due to its antiproliferative and pro-differentiating effects. However, as we show in this study, GBM cell lines fail to respond to the antiproliferative effects of $1,25(OH)_2D_3$. Moreover, *in vitro* treatment of GBM cells with $1,25(OH)_2D_3$, did not result in a modulation of the expression of key components of the Notch-signaling pathway. The reason for $1,25(OH)_2D_3$-resistance in GBM cell lines is unknown. Therefore, it was the aim of this study to investigate whether epigenetics mechanisms, that can modular gene transcription *via* control of chromatin Status, modulation of RNA polymerase II recruitment and other specific mechanisms, are of importance for the abrogation of vitamin D signaling in vitamin D resistant GBM cell lines. We used the histone deacetylase inhibitor (HDACI) TSA and the DNA-methyltransferase inhibitor (DNMTI) 5-aza to elucidate the effects of protein acetylation and of DNA hypermethylation on $1,25(OH)_2D_3$-induced effects on cell proliferation, respectively. As expected, treatment with TSA or 5-aza exerted dose- and time-dependent antiproliferative effects on GBM cell lines. However, the resistance of GBM cell lines against $1,25(OH)_2D_3$ could not be restored by co-treatment with TSA or 5-aza. In contrast, antiproliferative effects of TSA and 5-aza were partially antagonized by concomitant treatment with $1,25(OH)_2D_3$, indicating a protective effect of $1,25(OH)_2D_3$ against the antiproliferative effects of TSA and 5-aza in GBM cell lines. In conclusion, our findings point at a differential expression of key components of Notch-signaling pathways in GBM cell lines that may be of importance for the growth characteristics of GBM. Our findings do not support the hypothesis that modulation of Notch-signaling pathways by 1,25-dihydroxyvitamin D_3 may regulate growth of GBM cell lines.

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