The role of active Notch signaling in GBM neurosphere cultures

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Introduction

- Glioblastoma multiforme (GBM) is the most common and aggressive primary brain tumor in adults.
- Brain cancer stem cells (bCSC) are neural stem cell (NSC)-like cells found in GBM and they can be established and maintained *in vitro* as non-adherent neurosphere cultures. bCSC might be responsible for tumor-initiation, -progression, treatment resistance and relapse.
- Notch signaling is important for maintaining an undifferentiated pool of normal NSC and in determination of cell fate and has moreover been indicated to play a functional role in GBM and thereof derived bCSC.

Objectives

Investigate the significance of Notch expression and activation in GBM neurosphere cultures

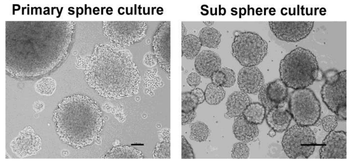
Methods

- Neurosphere cultures were established from human derived primary GBM xenografts and cultured in NB-media: Neurobasal™-A media supplemented with B-27, L-glutamine, EGF, bFGF and LIF. 029, 036 and 048 are three different primary GBM patient tumors and their corresponding xenograft tumors and neurosphere cultures.
- Notch inhibition was accomplished using the γ-secretase inhibitor DAPT dissolved in DMSO. Equal volumes of DMSO was used as a control.
- Notch activation was obtained by transiently transfecting the cells with pcDNA3.1(+)-i.c.Notch-1 plasmid, containing the intracellular Notch-1 domain (ICN-1), by Lipofectamine. Empty pcDNA3.1(+) plasmid (Mock) was used as a control.
- Protein expression was determined by Western blot analysis (WB) or immunocytochemical (ICC) labeling.
- mRNA expression was analyzed by Quantitative Real-Time Polymerase Chain Reaction (q-RT-PCR).

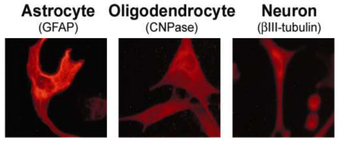
Results

Human GBM cells display bCSC characteristics and express Notch-1

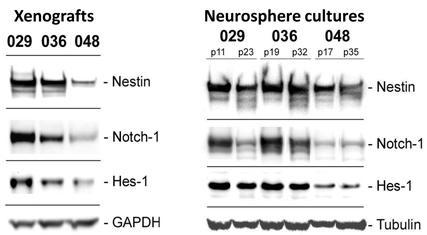
A: Sphere formation



B: Differentiation



C: Basal protein expression



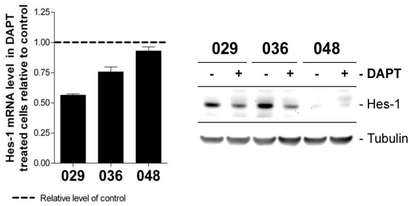
A: Neurospheres were formed from GBM xenograft cells and this sphere forming ability was maintained in later passages, indicating self-renewal.

B: All cultures were able to differentiate into cells expressing glial and neuronal markers, pointing out that multipotent cells are present in the neurosphere cultures.

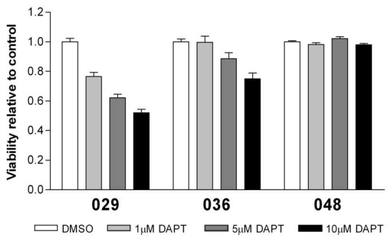
C: WB detection of the Notch-1 receptor, the Notch downstream target Hes-1 and the NSC marker Nestin shows that in particular Hes-1 expression is maintained from xenograft tissue throughout several passages of *in vitro* culturing.

The effect of Notch blockade is more pronounced in cultures with high Notch activity

A: q-RT-PCR and WB detection of Hes-1 in neurosphere cultures treated with DAPT



B: Viability analysis of neurosphere cells treated with DAPT



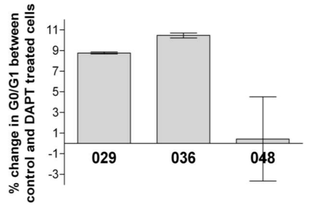
A: DAPT treatment, 5μM, led to down regulation of Hes-1 mRNA and protein, verifying inhibition of Notch signaling and thus diminished Notch activity.

B: MTT assay showed that DAPT treatment has a functional effect on the neurosphere cells as it hampered viability.

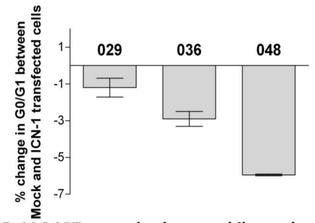
Results

GBM neurosphere cell cycle distribution is distorted upon Notch modulation

A: Cell cycle analysis on DAPT treated neurosphere cells



B: Cell cycle analysis on ICN-1 transfected neurosphere cells

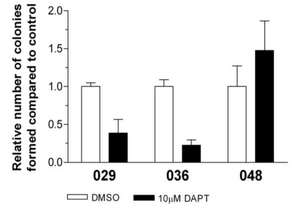


A: The G0/G1 population was expanded in the 5μM DAPT treated cultures, while no change was observed in the number of dead cells when counted (data not shown), together implying cell cycle arrest.

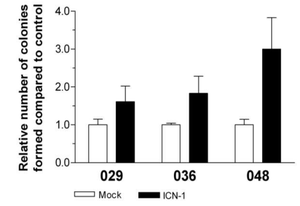
B: Upon transfection with ICN-1 the G0/G1 population was reduced when compared to mock transfected cells .

Notch modulation alters the *in vitro* tumorigenic potential of GBM neurosphere cells

A: Colony forming assay on DAPT treated neurosphere cells



B: Colony forming assay on ICN-1 transfected neurosphere cells



A: The potential to form colonies in soft-agar was clearly reduced in DAPT treated cultures reflecting reduced tumorigenic potential.

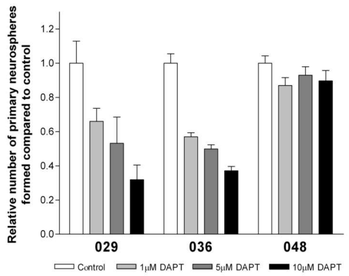
B: Notch activation, by ICN-1 transfection increased the colony forming potential, indicating improved tumorigenecity.

Discussion

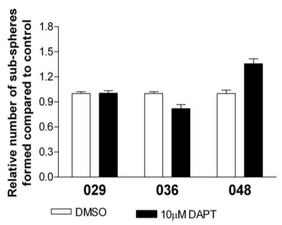
- Different tumorigenic features, such as cell viability, cell cycle distribution and colony formation showed sensitivity towards both Notch inhibition and activation.
- Active Notch signaling seems to be important for the bCSC features of GBM cells *in vivo* as the primary sphere potential was reduced upon Notch inhibition.
- As no obvious effect of Notch blockade was observed on the neurosphere bCSC features, such as self-renewal and differentiation, endogenous Notch activation might not be crucial for the *in vitro* bCSC characteristics. These characteristics could, however, be targeted by constitutively activating the Notch-1 receptor which led to increased self-renewal and dedifferentiation.
- Notch inhibition only affects cultures defined as having increased endogenous Notch activity

Modulation of Notch activity has a diverse effect on GBM neurosphere formation

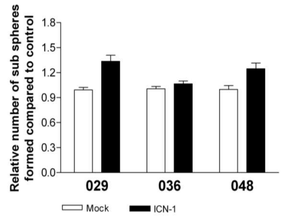
A: Primary sphere assay on acutely dissociated xenograft cells



B: Sub sphere assay on DAPT treated neurosphere cells



C: Sub sphere assay on ICN-1 transfected neurosphere cells

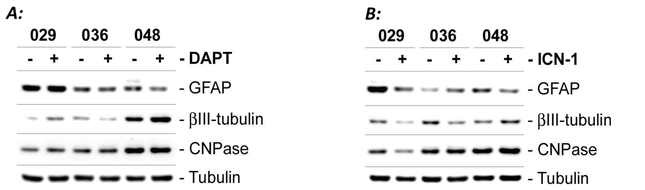


A: The ability to form neurospheres in primary culture was reduced in a DAPT concentration depended manner.

B: Neurosphere formation in later passage cultures was not diminished by Notch inhibition, which argue that active Notch signaling is not important for the self-renewing capacity of GBM neurosphere cells.

C: A tendency to an increase in neurosphere formation was detected in ICN-1 transfected neurosphere cells.

Notch modulation affects the differentiation level



WB detection of differentiation markers in neurosphere cells treated with 10μM DAPT (A) or transfected with ICN-1 (B) implies that Notch blockade has little effect on the differentiation level whereas artificial Notch activation leads to dedifferentiation of neurosphere cells.

Conclusion

Notch signaling plays an important role in GBM neurosphere cell growth

It is possible to affect the bCSC population by Notch pathway modulation

Notch targeted anti bCSC treatment could be feasible for GBM patients with high Notch pathway activation