Autocrine VEGFC-VEGFR2 signaling is of importance for the growth of Glioblastoma Multiforme

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Background

- **The Vascular Endothelial Growth Factor (VEGF)-A can together with a number of other VEGF variants activate the VEGF Receptor 2 (VEGFR2)**
- **VEGFR2 has in addition to be expressed on endothelial cells (where it is linked to angiogenesis through paracrine stimulation by VEGFA produced by the tumor cells) recently been shown also to be expressed by Glioblastoma Multiforme (GBM) tumor cells.**
- **However, although autocrine VEGFA-VEGFR2 signaling has been identified in GBM cells, contradicting results exist for the effects of inhibiting VEGFA and VEGFR2 respectively in GBM cells.**
- **This indicates that activation of VEGFR2 in GBM cells is not solely dependent on VEGFA.**

Aim

To examine if VEGFA is the only regulator of VEGFR2 in GBM tumor cells

Methods

- CPH017p4 and CPH036p6 are two different GBM cell cultures originating from two different primary tumors from GBM patients and their subsequent xenografts. Cells were cultured in Neurobasal-A media supplemented with B-27, L-glutamine, N2, EGF and bFGF.
- Cells were treated with SU1498 for inhibition of VEGFR2 phosphorylation, recombinant VEGF-A165 protein, Bevacizumab for inhibition of VEGFA, recombinant VEGFC protein and VEGFC-specific siRNA or non-specific scrambled (sc.) siRNA as control. The siRNA was delivered by plasmid transfection 24h prior to further experiments.
- Cell viability profiles were measured by MTT assay.
- Protein expression was determined by Western blot (WB) analysis while mRNA expression was measured by real-time quantitative PCR (Q-RT-PCR). Lysates from human dermal microvascular endothelial cells (HMVEC) and human dermal lymphatic endothelial cells (HDLEC) were used as controls.
- For establishment of tumor xenograft 100.000 CPH017p4 cells, which had been stably tranduced for Luciferin expression, were injected orthotopically into the brain of NMRI nude female mice. Cells had one day prior to injection been transfected with either VEGFC-siRNA or sc.-siRNA. Tumor development was followed by Bioluminescence imaging. Mice were sacrificed when they presented tumor related symptoms or 20% weight loss.
- Patient tumor material was collected during surgery at Rigshospitalet, Denmark, under approval of the Scientific Ethical Committee for Copenhagen and Frederiksberg (KF 01-034/04). Tumors were diagnosed as GBM according to the WHO 2000/2007 guidelines.
- Statistics: One sample t-test, setting the hypothetical value to 1, was used for analysis of Q-RT-PCR measurements. General linear model analysis was used for evaluation of in vitro growth curves, while survival analysis was performed using the Kaplan-Meier method and the log-rank test for comparisons between the treatment and the control group.

Results

VEGFR2 and VEGFA expression

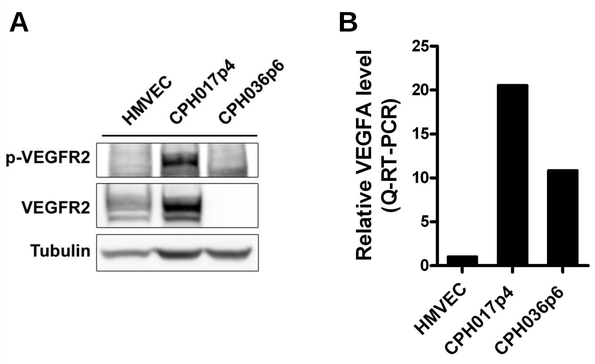

Figure 1: A) VEGFR2 and p-VEGFR2 protein levels (WB). B) VEGFA mRNA expression.

Role of VEGFA and VEGFR2 for the viability of GBM cells

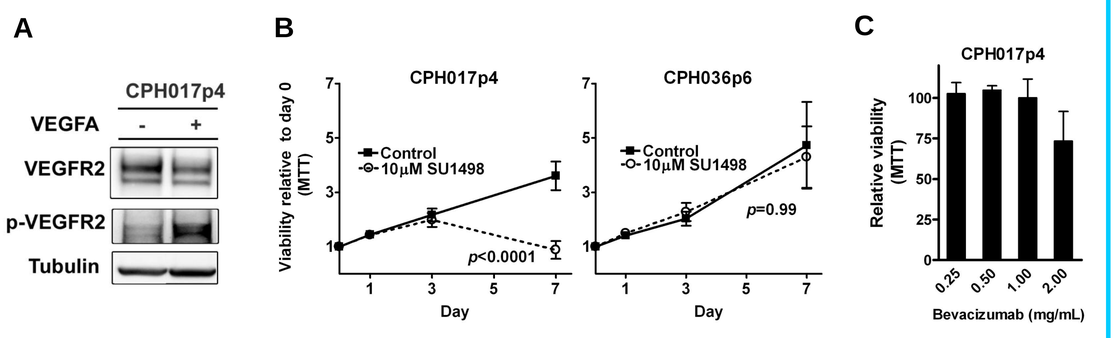

Figure 2: A) VEGFR2 and p-VEGFR2 protein level in CPH017p4 cells following treatment with VEGFA (40ng/mL in 15 min). B) Viability of cells treated with SU1498 over 7 days. C) Viability of CPH017p4 cells following treatment with Bevacizumab for 7 days.

VEGFC expression and its effect on p-VEGFR2 and cell viability

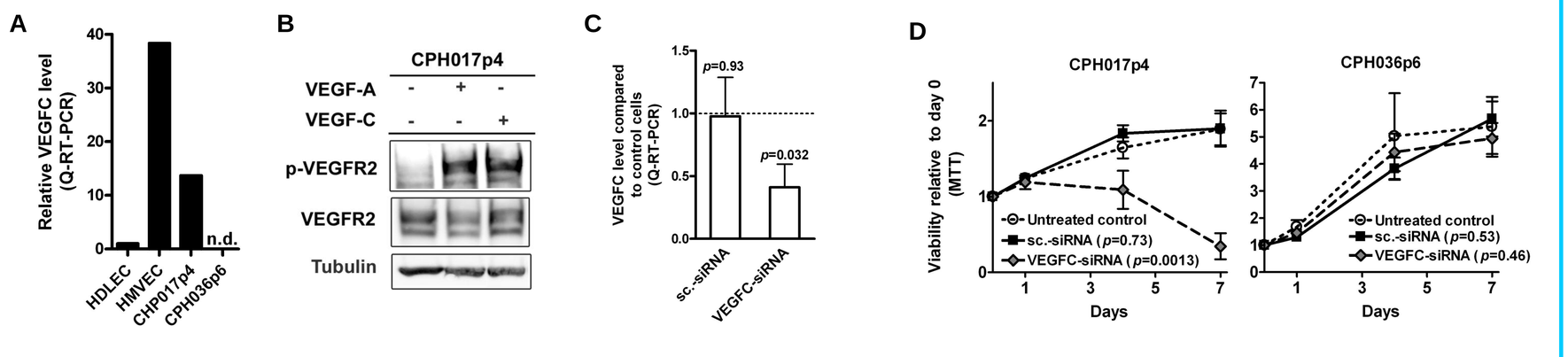

Figure 3: A) VEGFC level in cell cultures. B) WB showing VEGFR2 and p-VEGFR2 protein levels in CPH017p4 cells following treatment with VEGFC (0.2µg/mL) and VEGFA (40ng/mL) for 15 min. C) VEGFC expression in CPH017p4 cells 24 h following transfection with VEGFC-siRNA or sc.-siRNA constructs. D) Viability of VEGFC siRNA transfected cells over 7 days.

Effect of VEGFC knockdown on *in vivo* growth of GBM cells

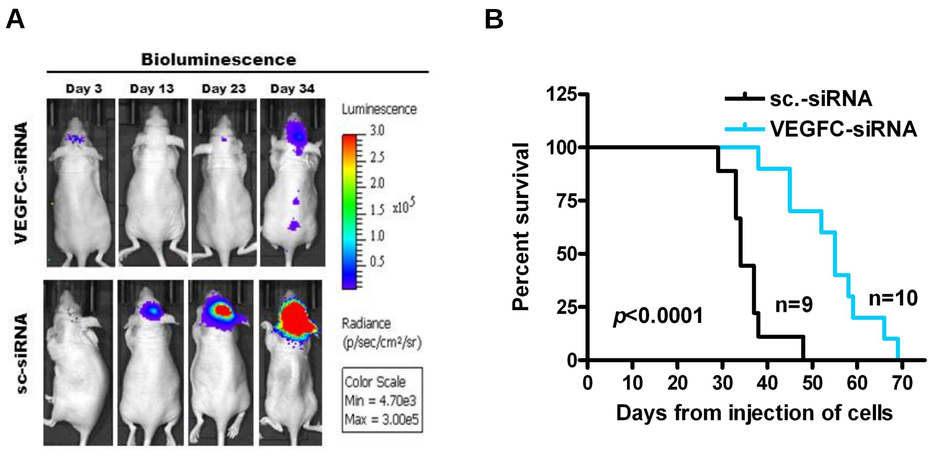

Figure 4: A) Representative pictures of bioluminescence imaging over time of mice injected with tumor cells transfected with VEGFC- or sc.-siRNA. B) Kaplan-Meier survival analysis of cumulative survival of VEGFC- and sc.-siRNA mice.

VEGFC expression in GBM patient tumors

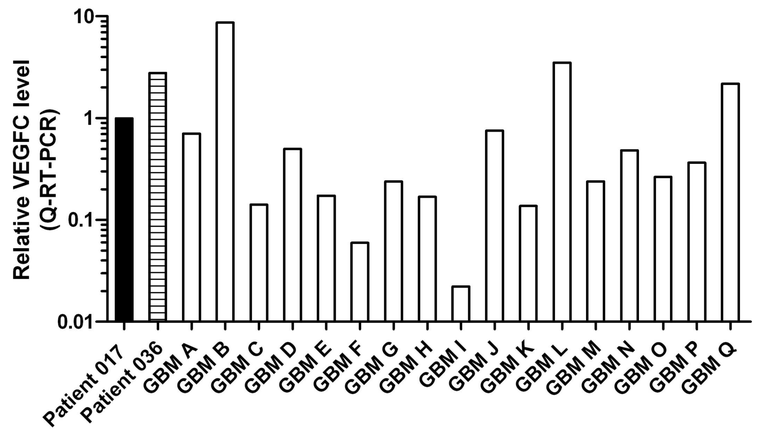

Figure 5: Relative VEGFC mRNA level in 19 GBM tumors. The Patient 017 and 036 tumors are the tumors from which the cell cultures CPH017p4 and CPH036p6 respectively were established. The GBM A-Q tumors are from 17 other GBM patients.

- **To study VEGFR2 regulation we used a VEGFR2-positive (CPH017p4) and a VEGFR2-negative (CPH036p6) GBM cell culture (Fig. 1A). Both cultures expressed VEGFA (Fig. 1B).**
- **VEGFR2 phosphorylation could be stimulated by VEGFA (Fig. 2A). However, while inhibition of VEGFR2 phosphorylation by SU1498 resulted in reduced proliferation of the VEGFR2-positive cells (Fig. 2B), Bevacizumab had only minimal effects on viability in these cells (Fig. 2C).**
- **The VEGFR2-positive cells were also positive for the VEGF variant VEGFC (Fig. 3A).**
- **Addition of VEGFC protein to the VEGFR2 positive cells induced VEGFR2 phosphorylation (Fig. 3B), while inhibition of VEGFC (by siRNA) reduced the *in vitro* growth (Fig. 3C-D).**
- **When injected into the brains of mice, VEGFC-siRNA transfected cells resulted in reduced tumor growth (Fig. 4A) and increased survival compared to control cells (Fig. 4B).**
- **In a panel of GBM patient tumors all were positive for VEGFC expression, although the level was varying (Fig. 5).**

Conclusions

- **Like VEGFA, VEGFC can stimulate autocrine VEGFR2 activation in GBM cells**
- **VEGFC is of importance for cell viability and tumor growth in GBM cells**
- **VEGFC is a potential target for future GBM therapy**