The role of VEGFC for cell viability, tumor growth and bevacizumab resistance in Glioblastoma Multiforme

Michaelaen SR1, Nedergaard MK2, Urup T1, Olsen LR3, Kjaer A2, Perryman L3, Erler JT4, Lassen U1 and Poulsen HS1

1Department of Radiation Biology, The Finsen Center, Rigshospitalet, Copenhagen, Denmark. 2Department of Clinical Physiology, Nuclear Medicine & PET and Cluster for Molecular Imaging, Rigshospitalet and University of Copenhagen, Copenhagen, Denmark. 3Biotech Research and Innovation Center (BRIC), University of Copenhagen, Copenhagen, Denmark.

ABSTRACT
To examine if other factors than VEGFA is responsible for VEGF-R2 activation in GBM cells.

AIM
To examine if other factors than VEGFA is responsible for VEGF-R2 activation in GBM cells.

METHODS
CPH017p4 and CPH036p are GBM cell cultures originating from two different GBM patients. Cells were cultured in Neurobasal-media with B-27, l-glutamine, N2, EGF and PDGF.

Tumors consisted of SU1498, recombinant VEGF-A165 and VEGF protein, Bevacizumab and VEGF-specific siRNA or non-specific scrambled (sc) siRNA as control. siRNA was delivered by plasmid transfection 24h prior to experiments.

Cell viability was measured using MIT assay, mRNA expression by real-time quantitative PCR (Q-RT-PCR), protein expression with Western blot (WB). Lysates from human dermal microvascular endothelial cells (HDMEC) and human dermal lymphatic endothelial cells (HDLEG) were used as controls.

For establishment of tumor xenograft 100.000 CPH017p4 cells, which had been stably transduced for Luc7h expression, were injected subcutaneously into the brain of MMIV nude female mice. Tumor development was followed by Biotracer imaging. Mice were sacrificed when they presented tumor related symptoms or 20% weight loss.

Patient tumor material was collected during surgery at Rigshospitalet, Copenhagen, under approval of the Scientific Ethical Committee for Copenhagen and Frederiksborg (KF 01-304/04). Tumors were diagnosed as GBM according to the WHO 2000-2007 guidelines.

RNA sequencing library was prepared using Illumina Human HiSeq. RNA-Seq Library System from Nugen and sequenced on a Genome Analyzer 2000 platform as paired end reads 2x101 bases. Raw data were processed using Cufflinks 1.2.1 and TopHat 0.6.2 to generate falsi files. Sequencing reads were mapped to the human genome (hg19) using TopHat and expression of transcript was quantified using Cufflinks. Transcript counts were fitted to a generalized linear model using DESeq2.

Statistics: One sample t-test, setting the hypothetical value to 1, was used for analysis of Q-RT-PCR measurements while a two sample paired t-test was used analyzing the RNA sequencing results. 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
• A VEGFR2-positive (CPH017p4) and a VEGFR2-negative (CPH036p) GBM cell culture was used to study VEGFR2 regulation (Fig. 1A). Both cultures expressed VEGFA (Fig. 1B).
• VEGF2 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).
• When injected into the brains of mice, VEGFC-siRNA transfected cells resulted in reduced tumor growth (Fig. 4A) and increased survival (Fig. 4B) compared to control cells.
• In a panel of GBM patient tumors all were positive for VEGF expression (Fig. 5A). However, no significant changes were observed when examining VEGF expression in parallel tumor samples taken before and after Bevacizumb therapy (Fig 5B)

CONCLUSIONS
• Like VEGFA, VEGFC can stimulate autocrine VEGF-R2 activation in GBM cells.
• VEGFC is of importance for cell viability and tumor growth in GBM cells, but not directly associated with acquired resistance towards Bevacizumab.
• VEGFC is a potential target for future GBM therapy as it is generally expressed in GBM tumors.

Reference
1. VEGFR2-
2. VEGFR1-
3. VEGFR3-
4. VEGF-
5. Bevacizumab-