Endothelial cell proliferation is not affected by downregulation of choline kinase

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Introduction: The increase of cellular phosphocholine (PC) and total choline-containing compound is one of the most widely established characteristics of cancer. This elevation is closely related to malignant transformation, invasion, and metastasis [1-3]. We have previously shown that both transient transfection and stable expression of siRNA (siRNA-chk) and shRNA against choline kinase (Chk), the enzyme that converts choline (Cho) to PC, significantly reduced proliferation in breast cancer cells [4] and tumors [5]. The downregulation of Chk in nonmalignant MCF-12A cells resulted in an almost negligible effect on PC and proliferation [6]. Endothelial cells are a key component of vasculature and are exposed to agents that are delivered systemically. They also play a major role in tumor vascularization and metastasis. To investigate the influence of transfection of siRNA-chk on endothelial cells, we have examined the proliferation and PC levels of human umbilical vein endothelial cells (HUVEC) after transient siRNA-chk transfection and compared the results with human breast cancer cells (MDA-MB-231).

Methods. **Cell culture and siRNA transfection**: MDA-MB-231 (ATCC), metastatic human breast cancer cells were grown in RPMI-1640 medium supplemented with 10% FBS. HUVEC (VEC tech.) were grown in MCDB-131 complete medium. Cells were transiently transfected with 100 nM siRNA targeting chk-α (siRNA-chk) for 48 hours using DhamaFECT (Thermo Fisher scientific Inc.). **NMR study**: MDA-MB-231 cells and HUVEC were transfected with siRNA-chk for 48 hours and water-soluble as well as lipid extracts were obtained from no treatment control, DhamaFECT and siRNA-chk-treated cells using the dual-phase extraction method [4]. Fully relaxed ^{1}H NMR spectroscopy of the water-soluble phase was performed on a Bruker Avance 500 spectrometer. Signal integrals of the phosphocholine (PC) peak were quantified as mM relative to cell number, cell volume (MDA-MB-231: 2050 μm^3 and HUVEC: 4530 μm^3), and an internal concentration standard. More than 10^7 cells were harvested for cell extraction. **mRNA quantification**: RNA was isolated at 48 hours post transfection using a Qiagen kit. Quantitative real-time PCR (q-RT-PCR) was performed using iQ SYBR Green Supermix and gene-specific primers in the iCycler real-time PCR detection system (Bio-Rad), with cDNA which was synthesized using qScript (Quanta Bioscience). **Immunoblot analysis**: 40μg of protein from cell lysates at 48 hours post transfection were resolved on 7.5% acrylamide gel using a polyclonal Chk antibody (custom made) and a monoclonal GAPDH antibody (Sigma). **Proliferation assay**: Cells were transfected with siRNA for 48 hours, changed to culture medium and cultured another 3 days, following which an MTS assay (Promega) was performed.

Results and Discussion: After siRNA-chk transfection, Chk mRNA levels of MDA-MB-231 and HUVEC were comparable (Figure 1). Basal levels of Chk mRNA and Chk protein in HUVEC were low to start with, and it was difficult to downregulate Chk in HUVEC further. Figure 2 shows significant downregulation of Chk protein in MDA-MB-231, and downregulation to a lesser extent in HUVEC after transfection of siRNA-chk. MTS assay result showed no significant reduction of proliferation in HUVEC after siRNA-chk transfection, while MDA-MB-231 showed a significant reduction of proliferation (Figure 3). The level of PC in HUVEC was about one tenth compared to MDA-MB-231 (Figures 4 and 5). PC level was significantly reduced in MDA-MB-231 after siRNA-chk transfection but there was about 10% reduction in HUVEC. These data suggest that Chk inhibition will not affect endothelial cells during systemic administration, nor will it affect tumor vasculature.

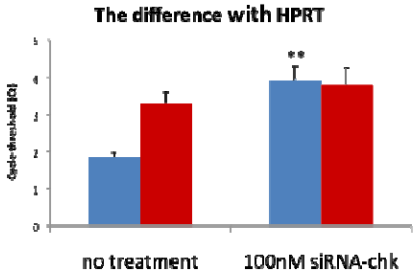

Figure 1: Cycle number required by q-RT-PCR for Chk mRNA in MDA-MB-231 cells ( ) and HUVEC ( ) compared with hypoxanthine phophoribosyltransferase (HPRT) mRNA. Values are mean ± SE, ** represents P < 0.01 *vs* no treatment, n=3.

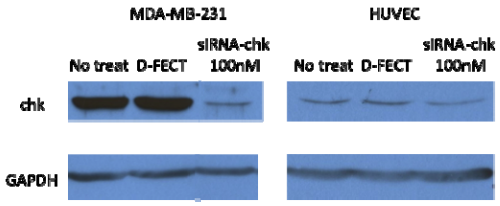

Figure 2: Chk protein expression levels in MDA-MB-231 cells and HUVEC. 40 μg of protein was loaded on 7.5% reducing SDS-PAGE gel. GAPDH: loading control.

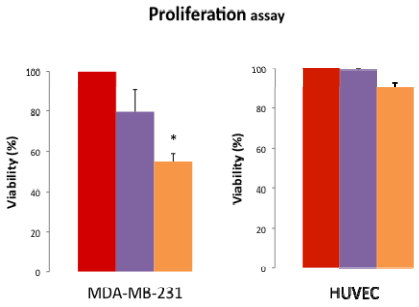

Figure 3: Cell viability/proliferation as determined by MTS assay in MDA-MB-231 and HUVEC cells treated with DharmaFECT ( ), 100nM negative siRNA ( ) and 100nM siRNA-chk ( ). Values are mean ± SE. * represents P<0.05 *vs* DharmaFECT, n=3.

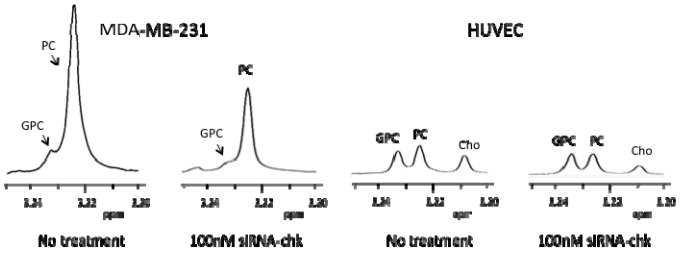

Figure 4: (a) Representative ^{1}H NMR spectra of 3.20-3.25 ppm region of MDA-MB-231 cells and HUVEC. Cho: freee choline; GPC: glycerophosphocholine.

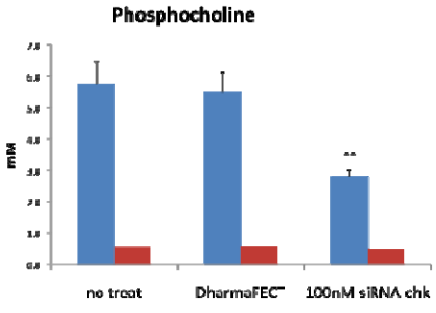

Figure 5: Levels of phosphocholine quantitated from ^{1}H MR spectra of MDA-MB-231 ( ) and HUVEC ( ). Values are mean ± standard error (SE).
** represents P < 0.01 *vs* no treatment.

References and Acknowledgements: [1] Aboagye E *et al*, *Cancer Res*, 59, 80 (1999); [2] Ackerstaff E et al, *Cancer Res,* 61, 3599 (2001);); [3] Ramirez de M *et al*, *Oncogene,* 21,4317 (2002); [4] Glunde K *et al*, *Cancer Res*, 65, (2005); [5] Krishnamachary B *et al*, *Cancer Res*, 69, (2009); [6] Mori N *et al*, *Cancer Res*, 67, (2007).
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