The Interdependence of Choline Kinase and Phospholipase D: Adaptation Mechanisms in Choline Phospholipid Metabolism of Human Breast Cancer Cells

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Introduction: MRS studies have consistently detected an elevation of PC and total choline containing compounds in human lung, colon, prostate, and breast tumors as well as derived epithelial cell lines [1, 2]. Enzymes in the choline phospholipid metabolism pathway therefore present unique targets to exploit for cancer treatment. Unlike pharmacological inhibitors, siRNA targeting has several advantages over other traditional therapeutic methods as siRNA can be generated to silence expression of any single or multiple genes. Choline kinase (Chk) is a cytosolic enzyme that catalyzes the phosphorylation of choline (Cho) to phosphocholine (PC) by ATP in the presence of magnesium. Over-expression of Chk-α has been observed in breast, prostate and lung cancers [3, 4], making it an obvious choice for targeting. We investigated siRNA-mediated down-regulation of Chk-α in human breast cancer cells, and observed significant down regulation of Chk-α (Figure 1A) and PC (Figure 1B). Down-regulation of Chk-α resulted in a significant reduction of cell proliferation and increased differentiation in highly invasive MDA-MB-231 human breast cancer cells after siRNA-Chk transfection [5].

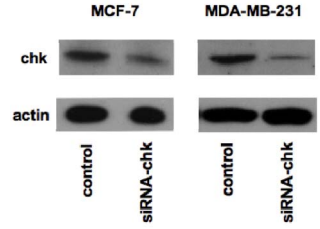

Figure 1A. Chk protein levels determined by immunoblot assay in MCF-7, and MDA-MB-231 control and siRNA-Chk treated cells. 50μg of protein from each cell line was loaded on a 10% reducing SDS-PAGE gel. β-actin protein levels were used for equal loading assessment. From [6].

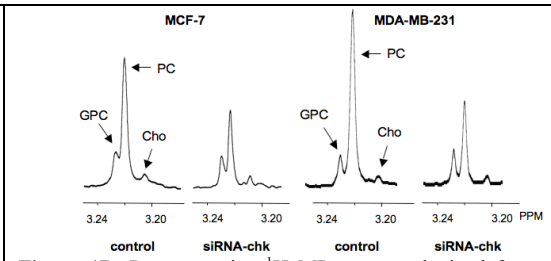

Figure 1B. Representative ^{1}H MR spectra obtained from water-soluble cell extracts of MCF-7, and MDA-MB-231 control and siRNA-Chk treated cells. Spectra were acquired on a Bruker Avance 500 spectrometer and are expanded to display signals from the PC, GPC, and Cho region only. GPC: glycerophosphocholine; PC: phosphocholine; Cho: free choline. From [6].

Two other potential targets are phosphatidylcholine-specific phospholipase C (PC-PLC) and PC-phospholipase D (PLD), subsequently called PLC and PLD. The gene sequence for PLC is currently not identified but may become available in the near future. PLD on the other hand is well characterized and increased PLD expression has been observed in several tumors [7]. PLD is known to regulate cell proliferation and survival. Two mammalian isoforms of PLD, PLD1 and PLD2 have currently been identified [8]. PLD over-expression has also been found to result in increased matrix metalloproteinase-2 expression and an increase of invasion [8]. Inhibitors of PLD have also been investigated as cancer therapeutic agents [7]. Both PLD1 and PLD2 exhibit unique regulatory properties. Unlike PLD2, which is constitutively expressed, basal expression of PLD1 is very low and is activated by G proteins such as ARF, Rho and Rac. Elevated PLD1 has been reported in gastric, renal, colon and breast carcinoma [7]. Elevated PLD activity and PLD1 protein have been reported to generate rapamycin resistance in MDA-MB-231 cells indicating that mTOR pathways may be affected in cancer cells with higher PLD1. We characterized PLD1 in metastatic MDA-MB-231 cells, less metastatic MCF-7 cells, and nonmalignant MCF-12A cells and used siRNA to down-regulate PLD1 in these cells. The effects of Chk-α downregulation on PLD1 expression and the effects of PLD1 downregulation on Chk-α expression were determined.

Methods: MCF-7 and MDA-MB-231 cells were transiently transfected either with siRNA targeting Chk-α or PLD1. Forty-eight hours post-transfection cells were harvested for protein and RNA. To determine the effective knock-down of Chk-α or PLD, about 30μg of protein was resolved on 7.5% acrylamide gel, transferred to nitrocellulose membrane and probed with antibody specific to either Chk-α or PLD1.

Results and Discussion: Chk and PLD1 protein levels and their downregulation following treatment with siRNA were determined with western blots and the message was quantified with q–RT-PCR (data not shown). As shown in Figure 2, downregulation of Chk resulted in an increase of PLD1 in MDA-MB-231 cells stably expressing shRNA against Chk or transiently transfected with Chk siRNA (similar data were obtained for MCF-7 cells). PLD1 levels were highest in the metastatic MDA-MB-231 cells (Figure 3A). Effective downregulation of PLD1 was achieved with transient PLD1 siRNA transfection (Figure 3B), which resulted in an increase of Chk in these cells (Figure 3C). These data demonstrate the adaptability of cancer cells, the interdependence of Chk and PLD1, and the importance of multiple targeting. These data support the need for multiple targeting of metastatic cancer cells with both Chk and PLD1 siRNA, to eliminate any adaptive compensatory effects that would allow cancer cells to survive. We previously observed a similar compensatory effect of PC-PLC upregulation and activation following Chk knockdown [9]. PC-PLC may provide another siRNA target once the gene is identified.

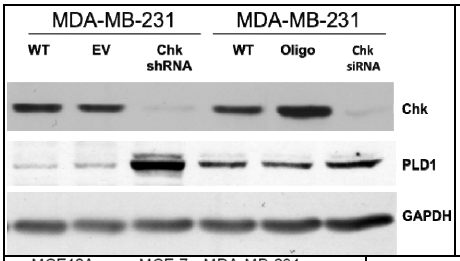

Figure 2. The effect of Chk downregulation on PLD1 in MDA-MB-231 breast cancer cells was determined using cells stably expressing shRNA against Chk or cells transiently transfected with Chk siRNA. In both cases an increase of PLD1 expression was detected. We are currently exploring the role of this PLD1 increase in the residual PC signal observed (Figure 1B) following Chk downregulation.

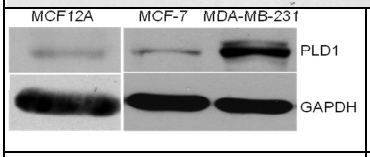

Figure 3A. Immunoblots showing PLD1 protein expression in breast cell lines with increasing malignancy. 30μg of protein was probed with rabbit polyclonal anti-PLD1 antibody (upper panel) and mouse monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody as a loading control (lower panel).

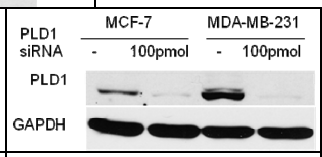

Figure 3B. Representative western blot showing PLD1 protein downregulation following transfection of siRNA against PLD1 in MCF-7 and MDA-MB-231 cells (upper panel). GAPDH as a loading control is shown in the lower panel.

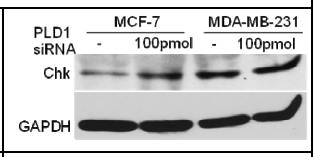

Figure 3C. Representative western blot showing increased Chk protein expression following transfection of siRNA against PLD1 in MCF-7 cells and MDA-MB-231 cells (upper panel). GAPDH as a loading control is shown in the lower panel.

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