Free Choline Influences Adaptation Mechanisms in Choline Phospholipid Metabolism of Human Breast Cancer Cells

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Introduction: A hallmark of cancer is an increase of cellular phosphocholine (PC) and total choline-containing compounds (tCho), which are closely related to malignant transformation, invasion and metastasis [1]. Enzymes in choline metabolism present attractive targets that can be exploited for treatment [2]. Two of these enzymes, choline kinase (Chk-α) and phospholipase D1 (PLD1) are closely associated with increased malignancy [1, 3]. Here we have shown that these two enzymes are interdependent. Downregulation of Chk-α with siRNA resulted in increased PLD1 expression, and downregulation of PLD1 resulted in increased Chk expression, typifying the ability of cancer cells to adapt. To further understand mechanisms underlying this interdependence, we characterized Chk and PLD1 expression at different concentrations of free choline. We observed a reduction in the increase of PLD1 expression following Chk downregulation with higher concentration of free choline, suggesting that free choline plays a role in mediating the increase of PLD1 observed following Chk downregulation.

Methods: MDA-MB-231 cells were transiently transfected either with siRNA targeting Chk-α (Chk-siRNA), PLD1 (PLD1-siRNA), or both. Transfected cells were maintained in normal RPMI 1640 media (21.5 μM of choline) or in RPMI with a five-fold increase of choline chloride (107.5 μM). Forty-eight hours post-transfection cells were harvested for protein and RNA. To determine the effective downregulation of Chk-α or PLD1, about 30 μg of protein was resolved on 7.5% acrylamide gel, transferred to nitrocellulose membrane, and probed with antibody specific to either Chk-α or PLD1. Effective downregulation was also assessed by quantitative real-time polymerase chain reaction (q-RT-PCR). High-resolution 1H MR spectroscopy of extracted water-soluble metabolites of MDA-MB-231 cells transfected with siRNA against Chk-α, PLD1, or both was carried out using a Bruker 500 MHz spectrometer. Metabolites were integrated and quantified relative to the signal from a known standard.

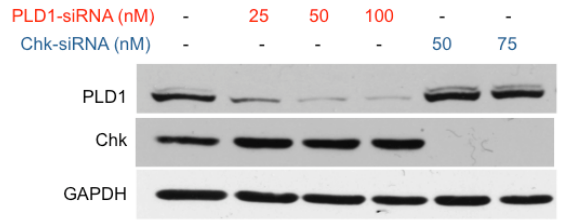

Figure 1: Effect of PLD1 downregulation on Chk-α and effect of Chk-α downregulation on PLD1 in MDA-MB-231 cells.

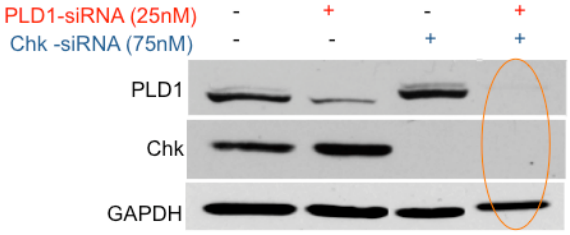

Figure 2: A combination of 25 nM PLD1-siRNA and 75 nM Chk-siRNA was effective in downregulating both Chk and PLD1 (orange outline) in MDA-MB-231 cells.

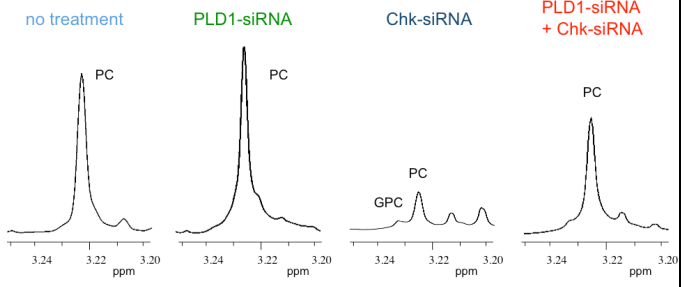

Figure 3: High-resolution spectra from MDA-MB-231 cells (left to right) untreated, treated with 25 nM PLD1-siRNA, treated with 75 nM Chk-siRNA, and treated with a combination of 25 nM PLD1-siRNA and 75 nM Chk-siRNA.

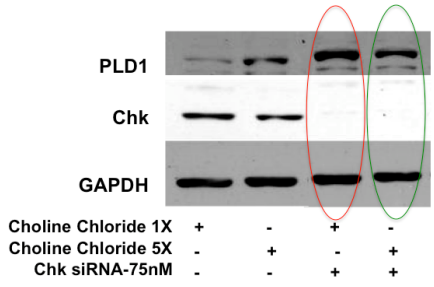

Figure 4: Representative immunoblot (from four separate experiments) of MDA-MB-231 cells showing PLD1 and Chk expression without and with addition of five-fold excess of choline. A compensatory increase of PLD1 is apparent following treatment with 75 nM Chk-siRNA for cells maintained in RPMI (outlined in red) that is significantly attenuated in cells maintained with 107.5 μM choline chloride (outlined in green). GAPDH is used as a loading control.

Results and Discussion: As shown in Figure 1, downregulation of Chk resulted in an increase of PLD1 in MDA-MB-231 cells transiently transfected with Chk-siRNA, and downregulation of PLD1 resulted in an increase of Chk-α. Following titration of Chk-α and PLD1 downregulation with different siRNA concentrations, we determined that a combination of 25 nM PLD1-siRNA and 75 nM Chk-siRNA resulted in an effective downregulation of both enzymes as shown in Figure 2. However, as shown in Figure 3, PC levels following combined Chk- and PLD1-siRNA treatment cannot be explained by the compensatory effect of PLD1 downregulation on Chk-α alone. Extensive work by Podo and colleagues [4] has established that PC-PLC also contributes to PC levels in cancer cells and tumors, and it is possible that PC-PLC may also change following Chk-α or PLD1 downregulation. We previously observed a compensatory effect of PC-PLC upregulation and activation following Chk knockdown [5]. As shown in Figure 4, increased concentration of free choline significantly attenuated the compensatory increase of PLD1 following Chk knockdown, identifying free choline as a mediator in this pathway. It is increasingly evident that in a complex disease such as cancer, it is important to understand the network of pathways that participate following downregulation of a specific target. These participating pathways provide novel opportunities to target in concert with the primary target to achieve improved control and minimize compensatory responses that allow cancer cells to survive or adapt. This is especially important if the downregulation of one 'oncogenic target', *e.g.* Chk-α, results in the upregulation of another, *e.g.* PLD1.

Acknowledgements: This work was supported by NIH P50 CA103175. **References: 1**. Glunde, K.. *et al.* Expert Rev Mol Diagn, 2006; **2**. Bañez-Coronel M. et al., Curr Cancer Drug Targets, 2008; **3**. Toschi, A. *et al.*, Cancer Letters,2010; **4**. Iorio, E. *et al.,* Cancer Res., 2010; **5**. Glunde, K. *et al.*, ISMRM 2008, Abstract 244.