Hypoxia and HIF silencing dysregulates total choline, CD44 expression, and metastatic burden in MDA-MB-231 human breast cancers

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Introduction: Hypoxia is frequently encountered in cancer and results in the stabilization of hypoxia inducible factors (HIF-1/2) that transcriptionally activate genes involved in invasion, metastasis, metabolism, and in the adaptation of cancer cells to their microenvironment. In breast cancer, stem-like breast cancer cells that survive, repopulate, and metastasize to distant locations, have elevated expression of CD44. We observed elevated expression of CD44 in hypoxic tumor regions, and identified HIF-1α as a regulator of CD44 in breast cancer cells under hypoxic conditions [1]. Hypoxia has also been implicated in increasing the activity of choline kinase (Chk) - an enzyme responsible for the elevated phosphocholine (PC) and total choline [2] levels that are consistently observed in cancer. Here, using non-invasive ^{1}H magnetic resonance spectroscopy (MRS) of intact cells and tumors, we have established the importance of HIF in reducing total choline level and metastatic tumor burden, and we have identified a role for CD44 in establishing lung metastasis.

Methods: MDA-MB-231 human breast cancer cells expressing shRNA against HIF-1α and HIF2 α were established as previously described [1] and cell perfusion was performed as previously described [3]. *In vivo* MRSI studies were performed using tumors derived from MDA-MB-231 cells and sub-lines silenced for HIF (-1α, -2α or both (double silenced)), implanted in the mammary fat pad of female severe combined immunodeficient (SCID) mice. The metastatic burden potential of the different cell lines was explored by injecting them intravenously in female SCID mice. MR experiments on volume-matched tumors were performed on a Bruker 4.7T MR spectrometer using a home-built RF resonator. MR spectra were processed and analyzed with an in-house IDL program. Paraffin embedded adjacent sections of lung samples were stained for hematoxylin and eosin (H&E) and CD44. High-resolution digital scans of the stained sections were obtained using ScanScope (Aperio, Vista, CA). Images were processed, and nuclei and membrane intensity quantified using ImageScope software and algorithm supplied by the manufacturer.

Results and Discussion: HIF silencing in MDA-MB-231 cells significantly delayed tumor growth in mice (Figure 1A). ^{1}H and ^{31}P MR spectra of double silenced cells showed decreased total choline (tCho) and PC as compared to parental control cells (Figure 1B). Similar trends were observed *in vivo* with 1h MRSI. A statistically significant decrease in tCho was observed in tumors derived from double silenced cells (Figures 1C-D). Western blot analysis of tumors detected a decrease in Chk expression in double silenced tumors. Silencing HIF-1α, -2α or both resulted in a significant reduction of metastatic lung burden in mice (Figures 2A-B). Additionally, HIF-2α silencing was more effective at reducing lung colonization than HIF-1α, while silencing both was the most effective. Although metastatic burden decreased in HIF-1α silenced cells, the percentage of cells with high CD44 expression in the metastatic foci was comparable to that in the wild type or empty vector (EV) foci (Figure 2C). These data identify the importance of targeting HIF and CD44 to prevent lung colonization and disrupt the metastatic cascade.

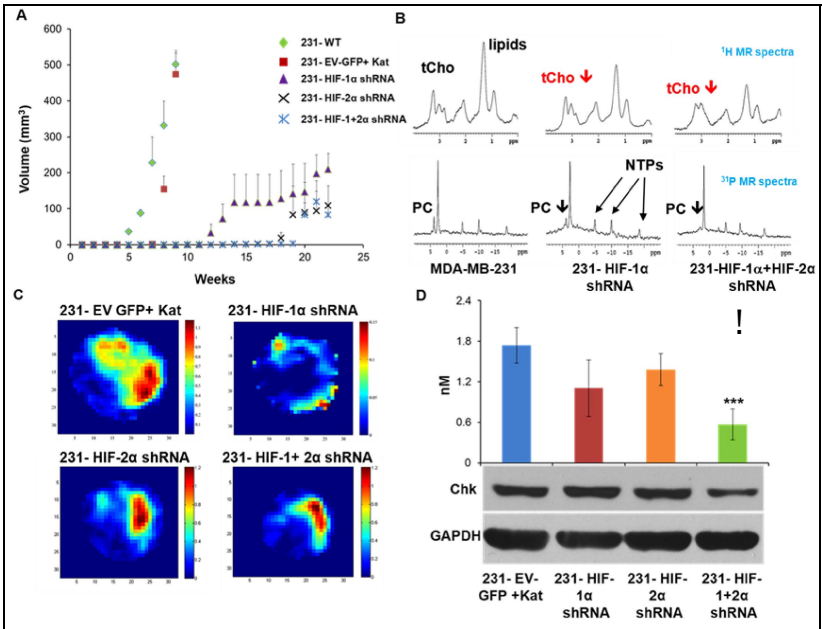

Figure 1: Metabolic changes in HIF silenced MDA-MB-231 cells. (A) Tumor growth curve of HIF silenced cells. **(B)** Representative ^{1}H and ^{31}P MR spectra showing tCho and PC levels in HIF silenced cells. **(C)** Representative *in vivo* MRS images of tumors derived from HIF silenced cells showing tCho distribution. **(D)** Quantification of tCho showing statistically significant reduction in tumors obtained from double silenced cells. Values represent Mean ± SE of 5 animals per group. *** $p \leq 0.0005$. Western blot showing Chk expression from representative tumor extract from each group. Bottom lane shows glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression as control.

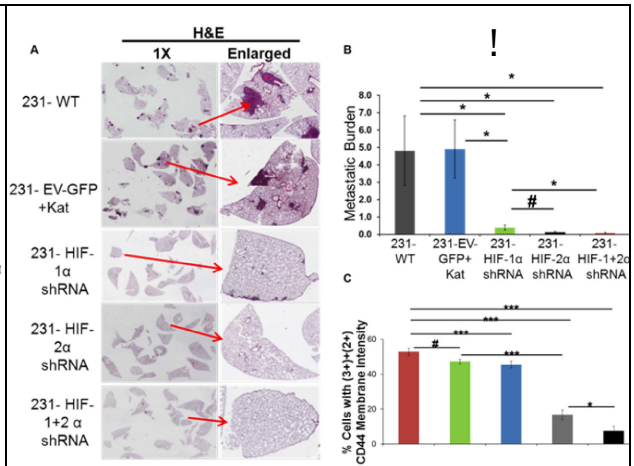

Figure 2: Metastatic foci and CD44 expression. Representative lung sections obtained from animals injected with HIF silenced MDA-MB-231 cells and stained for H&E. Arrow in red represents the enlarged field of view. Images were captured using Aperio ScanScope. **(B)** Metastatic burden was calculated from the nuclear staining intensity of H&E stained sections as the ratio between the areas of the foci in which at least 20 or more cells are positive for 3+ (high intensity) nuclear staining and total area of the lung per each lung sections/ mouse. **(C)** CD44 membrane intensity was also calculated as above, but scored for CD44 cell surface expression. Values represent Mean ± SE of 5 sections. # $p \leq 0.08$, * $p \leq 0.06$, *** $p \leq 0.0005$.

References: **1**. Krishnamachary B. *et al.,* PLoS One, 2012; **2**. Glunde, K., *et al.,* Cancer Res, 2008; **3**. Ackerstaff E. *et al.,* Neoplasia. 2007.

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