HIF-1α and HIF-2α Double Silenced MDA-MB-231 Human Breast Cancer Cells show reduced Invasion and Degradation of the Extracellular Matrix

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Introduction: Hypoxia is encountered in most solid tumors and is associated with poor prognosis [1]. Cancer cells display an adaptive response to hypoxia through the activation of several genes. Activation of these genes is mediated through the binding of hypoxia inducible factor (HIF)-1 to hypoxia response elements (HRE) that results in increased transcription [2]. Among the genes activated are several proteolytic enzymes that play a role in invasion, metastasis and metabolism [3]. The MDA-MB-231 human breast cancer cell line is an aggressive and triple (estrogen receptor/progesterone receptor/Her-2 neu) negative human breast cancer cell line that is highly invasive and metastatic. Here we genetically engineered MDA-MB-231 cells to silence the expression of either HIF-1α (HIF-1α silenced) or both α isoforms of HIF (HIF-1α+HIF-2α), subsequently called doubly silenced cells. We investigated the ability of these cells to invade and degrade the extracellular matrix (ECM) under normoxic or hypoxic conditions in our MR compatible cell perfusion assay and determined metabolic changes in these cells.

Materials and Methods: Detailed description of the MR cell perfusion system can be found in Ackerstaff *et al.* [4]. Experiments were performed under normoxic or hypoxic conditions at oxygen tensions of 20% and 1% respectively. The oxygen tension was monitored using ^{19}F MR relaxometry of perfluorocarbon doped alginate beads that were interspersed within the layers of cancer cells. MR data were acquired on a 9.4 T MR spectrometer (Bruker, Billerica, MA) every 12 h over a period of 2 days. A chamber containing Matrigel® at a concentration of 8.8 mg/ml, which is part of the MR-compatible cell perfusion assay, was used to determine the degradation of ECM by the cancer cells. Degradation of ECM by cancer cells was determined at the 24 h time point relative to the initial time point from the proton images. Degradation of the ECM gel was estimated by drawing a region of interest (ROI) around the ECM gel region using NIH ImageJ software. The degradation index was defined as $(ROI_0-ROI_{24})/ROI_0$. One dimensional (1D) ^{1}H MR profiles of intracellular water were acquired along the length (z-axis) of the sample by diffusion-weighted (DW) MRI. These profiles were used to derive an invasion index by quantifying the number of cells invading into the ECM, as the signal from slow-diffusion water, which represents intracellular water, is directly proportional to the number of cells. Intracellular metabolite levels including total choline (tCho) *i.e.* signals from phosphocholine (PC) + glycerophosphocholine (GPC) + free choline, creatine/phosphocreatine (Cr/PCr), and lipids were derived from unlocalized DW ^{1}H MR spectra.

Results and Discussion: The immunoblots showing the reduced expression level of HIF-1α and HIF 2α in HIF silenced cells relative to control MDA-MB-231 cells are shown in **Figure 1a**. Representative T_1-weighted ^{1}H MR images demonstrating degradation of ECM gel by parental MDA-MB-231 and HIF-α silenced cells at various time-points under normoxia and hypoxia are shown in **Figure 1b**. The ECM gel was completely degraded by MDA-MB-231 cells by 48 h under normoxic conditions. Under hypoxic conditions, MDA-MB-231 cells completely degraded ECM within 24 instead of 48 h. HIF-1α silenced cells showed similar degradation as parental cells under normoxia and hypoxia (not shown). On the other hand doubly silenced cells showed significantly reduced degradation of ECM compared to parental cells under normoxia and hypoxia. **Figures 1c and 1d** show the degradation index and invasion index obtained from these cells. As shown in **Figure 1b** a significant increase in the degradation index in parental and HIF-1α silenced cells was observed with hypoxia, that did not occur in doubly silenced cells. These cells also showed significant decrease of degradation and invasion (**Figure 1d**) even under normoxic conditions compared to parental cells.

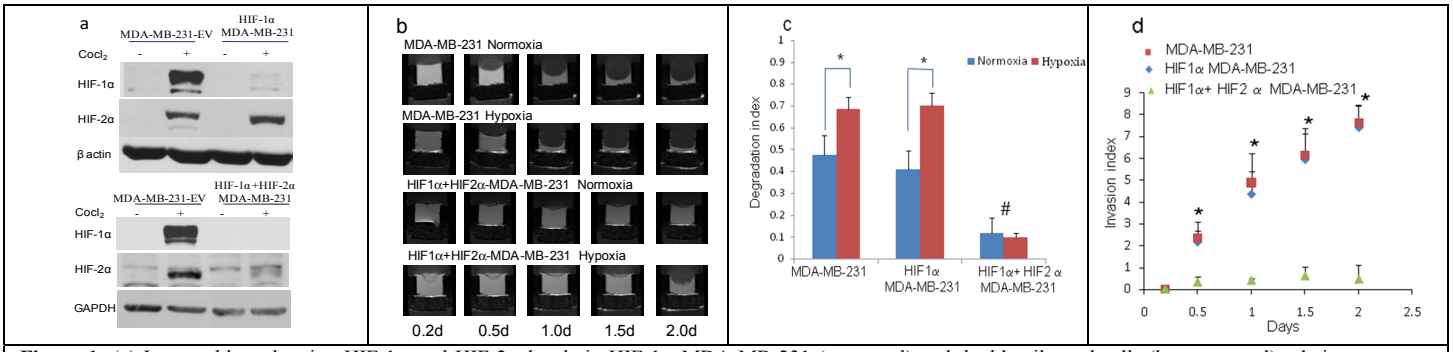

Figure 1: (a) Immunoblots showing HIF-1α and HIF-2α levels in HIF-1α MDA-MB-231 (top panel) and doubly silenced cells (bottom panel) relative to empty vector (EV) MDA-MB-231 cells. (b) Representative T_1-weighted ^{1}H MR images zoomed to display the region with the ECM chamber, showing degradation of ECM gel by MDA-MB-231 cells under normoxia and hypoxia. (c) Degradation index estimated from ECM gel degradation at 24 h relative to the initial time point (* p <0.01, n=5). # Represents significant decrease (p <0.01) compared to parental and HIF-1α silenced cells under normoxia and hypoxia (d) Invasion index *vs* time obtained from intracellular water signal over two days for parental and HIF knocked down cells under normoxia. (* p < 0.01, n=5). Values represents Mean ± SD.

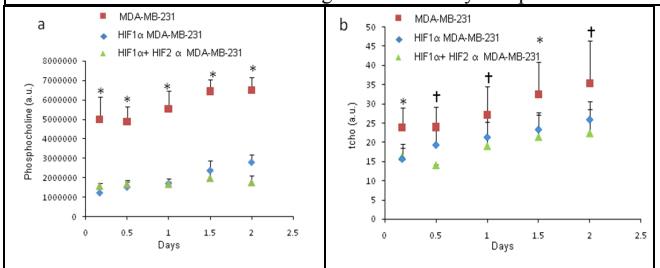

Figure 2: Quantification of data from (a) ^{31P} MR spectra and (b) ^{1}H spectra in arbitrary units demonstrating differences in tCho and PC (*p <0.05,†p <0.07). Values represents Mean ± SD.

This enhanced degradation of ECM under hypoxia may be driven by the upregulation of a number of proteolytic enzymes involved in ECM degradation [3]. The absence of any significant difference in ECM degradation between HIF-1α silenced and parental cells, coupled with a significant difference observed in the doubly silenced cells indicates the importance of HIF-2α in these cells, and is currently under investigation. Quantitative analyses of metabolites, shown in **Figures 2a and b**, demonstrate significantly decreased PC ($p<0.05$) and tCho ($p < 0.07$) in HIF-1α silenced and doubly silenced cells compared to parental cells, consistent with our earlier observations of transcriptional regulation of choline kinase by hypoxia [5]. These results suggest that *in vivo*, cells in or near hypoxic regions are likely to be more invasive and degrade the ECM, which can influence metastasis as well as drug delivery. These data also indicate that targeting HIF-1α alone is not sufficient to attenuate the invasiveness of these triple negative breast cancer cells under hypoxia and both HIF-1α and HIF-2α downregulation is required to attenuate or inhibit invasion and metastasis.

References: **1**. Ruan K *et al.,* J Cell Biochem 2009; 107:1053-62. **2**. Maxwell PH *et al.,* Proc Natl Acad Sci 1997;94:8104-9 **3**. Cassavaugh J *et al.,* Cell Biochem 2011;112-735-44. **4**. Ackerstaff E *et al.,* Neoplasia. 2007;9:222-35. **5** Glunde *et al* Cancer Res 2008;68:172-80. **Acknowledgements:** This work was supported by NIH RO1CA82337, R01CA73850 and P50CA103175. We thank Dr Dikoma Shungu and X Mao for XsOs software.