

Molecular characterization of the relationship between hypoxia, total choline and breast cancer stem cell markers

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Introduction: The discovery of cells with stem-like characteristics in several cancers, that are the most likely to be resistant to therapy and to lead to recurrence and metastasis [1], is offering new paradigms for understanding and treating tumor recurrence and metastasis [2]. Some of the molecular and functional markers that can be used to identify populations enriched with cells with stem-like characteristics are (i) CD44^{+/CD24^{low}} phenotype for breast cancer, (ii) exclusion of Hoechst 33342 (ABCG2 transporter/BCRP) and rhodamine-123 (MDR1), (iii) increased aldehyde dehydrogenase (ALDH1) activity, and (iv) tumor growth from low cell inoculums [1, 3-5]. The unique physiological environment of solid tumors is characterized by heterogeneous areas of poor blood flow [6], hypoxia [7], elevated total choline [8], high lactate [9] and low pH_e [10], which influence a wide-spectrum of phenotypic characteristics of tumors including progression, distant metastasis and response to therapy. Recent studies suggest that hypoxia provides a suitable niche for stem cells to maintain their precursor status. In tumors, hypoxia is also a major cause of radiation and chemo-resistance [11]. Here we have performed additional molecular imaging validation and molecular characterization of the role of hypoxia and choline metabolism in increasing stem-like characteristics in breast cancer xenograft cells and tumors.

Methods: Studies were performed with MDA-MB-231 tumors stably transfected with red-fluorescent tdTomato protein (RFP) expressed under control of the VEGF hypoxia response element (HRE). Xenografts were grown orthotopically in female severe combined immunodeficient (SCID) mice. MR experiments were performed with a Bruker horizontal bore 9.4T animal MR scanner using a home-built RF resonator. Fluorescence imaging of the tumor was performed *in vivo* with a Xenogen IVIS 200 system, and endpoint fluorescence imaging was performed with a fluorescence microscope using fresh 2-mm tumor slices prepared with a tissue slicer. For SPECT/CT imaging, mice were administered intravenously with 0.616 mCi of ¹²⁵I labeled anti-CD44 antibody in 0.17 ml of saline. At 48 h post injection, SPECT images of mice as well as tissue slices were acquired on a Gamma Medica X-SPECT scanner.

Additional studies were performed with empty vector (EV) MDA-MB-231 cells and these cells with HIF-1 α stably knocked down (Figure 2) to further understand the hypoxic response of stem-like breast cancer cell markers.

Results and Discussion: As before we observed an association between elevated total choline and hypoxia in tumors (Figure 1a) and a co-localization between increased CD44 expression and hypoxia (Figure 1b). We observed a significant increase of CD44, ABCG2 and ALDH1 mRNA levels following hypoxia induced by the hypoxia mimetic cobalt chloride (CoCl₂) in MDA-MB-231 cells (Figure 3). The increase of VEGF mRNA confirmed the induction of hypoxia. As anticipated, HIF-1 α mRNA did not increase under hypoxia (Figure 3), but we observed an increase of HIF-1 α protein under hypoxic conditions (Figure 2). In contrast CD44, ABCG2 and ALDH1 mRNA increase was significantly reduced in cells stably knocked down for HIF-1 α , following hypoxia (Figure 3). These data further support the role of hypoxia in increasing the expression of markers associated with stem-like breast cancer cells, and the importance of targeting hypoxia to minimize the burden of cells with stem-like characteristics in tumors.

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References: 1. Al-Hajj, M., *et al.*, Proc Natl Acad Sci U S A, 2003; 2. Al-Hajj, M., *et al.*, Curr Opin Genet Dev, 2004; 3. Ponti, D., *et al.*, Cancer Res, 2007; 4. Ho, M.M., *et al.*, Cancer Res, 2007; 5. Ginestier, C., *et al.*, Cell Stem Cell, 2007; 6. Feldmann, H.J., *et al.*, Strahlenther Onkol, 1999; 7. Vaupel, P., *et al.*, Cancer Res, 1991; 8. Glunde, K., *et al.*, Mol Pharm, 2006; 9. Walenta, S., *et al.*, Cancer Res, 2000; 10. Griffiths, J.R., Br J Cancer, 1991; 11. Brahimi-Horn, M.C., *et al.*, J Mol Med, 2007; 12. Khan, S.A., *et al.*, Clin Exp Metastasis, 2005.

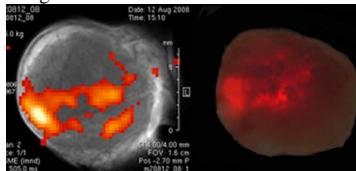


Figure 1a. Distribution of total choline (left) in an MDA-MB-231 HRE-RFP tumor (~250 mm³). Distribution of RFP in 2 mm slice corresponding to the 4 mm MRSI slice (right); the RFP image has not been warped to the MRS image but the association between total choline and hypoxia is evident.

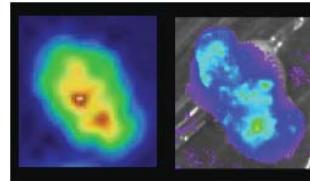


Figure 1b. SPECT image (left) from 2 mm fresh tissue slice of MDA-MB-231 tumor showing the distribution of CD44 expression. RFP image of this tissue section (right) obtained with Xenogen IVIS showing co-localization of fluorescence with high CD44 expression.

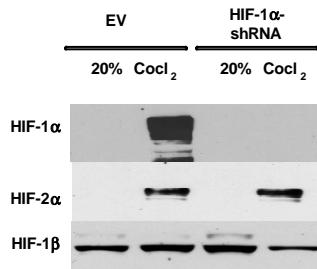


Figure 2. MDA-MB-231 empty vector (EV) cells or a transduced population of MDA-MB-231 cells expressing HIF-1 α shRNA were maintained in an incubator at 37°C with or without 200 μ M of the hypoxia mimetic CoCl₂. Resolved proteins were transferred to nitrocellulose membrane and probed with monoclonal antibody against HIF-1 α and re-probed with monoclonal antibody recognizing HIF-1 β .

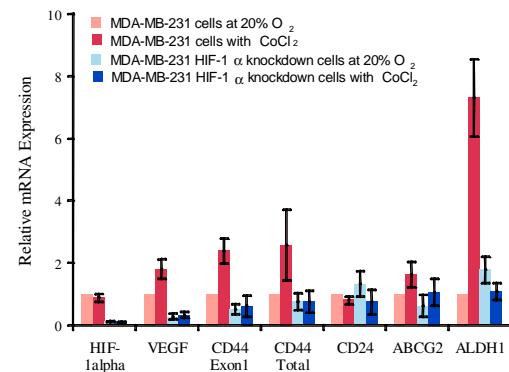


Figure 3. MDA-MB-231 cells and a transduced population of MDA-MB-231 cells stably expressing HIF-1 α shRNA were maintained in an incubator at 37°C with or without 200 μ M of the hypoxia mimetic CoCl₂. Twenty four hours later, total mRNA was isolated and cDNA prepared. Gene expression for VEGF and CD44 was assessed by q-RT-PCR using specific primers. Two different CD44 primers spanning Exon 1 or designed from published data (CD44 total [12]) were designed to recognize all isoforms of CD44. Relative mRNA expression is displayed normalized to values obtained for MDA-MB-231 cells maintained at 20% O₂. Values are Mean \pm S.D. obtained from three separate experiments.