Breast cancer cells can be rescued by Matrigel from the growth inhibitory effects of HIF-1α and HIF-2α silencing

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Introduction: Tumor microenvironments are frequently hypoxic and result in the stabilization of hypoxia inducible factors (HIF-1/2) that transcriptionally activate genes involved in invasion, metastasis, metabolism and angiogenesis [1]. The role of hypoxia and the contribution of HIF in the angiogenic switch leading to tumor progression and resistance to treatment is well documented [2]. This angiogenic phenotype in response to HIF activity is mediated through activation of vascular endothelial growth factor (VEGF)[3]. Most studies investigating the role of HIF in angiogenesis have focused on the gain of function of HIF. The noninvasive characterization of the loss of both isoform of HIFs (HIF-1α and HIF-2α) on tumor vascularization is relatively unexplored. Here we investigated the effect of HIF silencing on tumor growth in the presence or absence of Matrigel, a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells [4] that resembles the complex extracellular matrix (ECM) found in most tumors and determined its effect on tumor vasculature using noninvasive MRI.

Methods: MDA-MB-231 human breast cancer cells expressing shRNA against both HIF-1α and HIF-2α (231-HIF-1+α2shRNA) were established as previously described [5]. *In vivo* studies were performed using MDA-MB-231 breast cancer cells expressing an empty vector control (MDA-MB-231-EV) or HIF silenced cells (231-HIF-1+2α shRNA) orthotopically implanted in the mammary fat pad of female severe combined immunodeficient (SCID) mice. Tumor growth curves were obtained from cells inoculated either in 0.05 ml of Hanks balanced salt solution (HBSS) or together with Matrigel solution (8.8 mg/ml) (Sigma-Aldrich, St Louis, MO). All MRI studies were performed on a 9.4T Bruker Biospec preclinical horizontal bore scanner. 3D maps of vascular volume and permeability were obtained using a rapid gradient-echo sequence as previously described [6]. Briefly, tumor bearing mice were cannulated for injecting albumin-Gd-DTPA (0.5 g/kg) and placed in the magnet. A proton density (PD) image was acquired prior to contrast agent (CA) injection, using a 3D gradient echo sequence, with TE/TR = 1.5/10 ms and 3° flip angle. A quantitative M0 map was derived from this PD image, using a calibration constant determined from two gradient echoes images acquired separately at TE/TR = 1.5/10,000 ms with a 90° flip angle; and TE/ TR = 1.5/10 ms and a nominal 3° flip angle. T1-weighted images were acquired using a saturation-recovery 3D gradient echo with TE/TR = 1.5/5.0 ms, FOV 16x16x16 mm, matrix size 128x64x50 and 4 scans. A baseline image was collected before the intravenous administration of albumin-Gd-DTPA at 0.16 mmol (Gd)/kg, and dynamic contrast-enhanced images were collected for 30 min after the injection. At the end of the dynamic imaging studies, blood T1 was measured using inversion recovery. Data analysis was performed using in-house programs developed in the IDL language. 3D images were visualized using the ImageJ and Amira packages. All surgical procedures and animal handling were performed in accordance with protocols approved by the Johns Hopkins University Institutional Animal Care and Use Committee, and conformed to the Guide for the Care and Use of Laboratory Animals published by the NIH.

Results and Discussion: As shown in **Figure 1A**, exposure to hypoxia did not result in increased HIF-1 or 2 α protein expression in MDA-MB-231 cells expressing shRNA against both isoforms of HIF compared to empty vector cells. HIF silenced cells, when inoculated in the mammary fat pad of mice in the presence of Matrigel, clearly showed significantly improved growth advantage *in vivo* (**Figure 1B, lower panel**) compared to HIF silenced cells inoculated without Matrigel (**Figure 1B upper panel**). Empty vector cells also demonstrated an increase of growth rate with Matrigel, but this was not as dramatic as in HIF silenced cells. Previous studies in breast cancer have reported on the growth advantage provided by Matrigel [7].

Interestingly, HIF silenced tumors showed significantly increased vascular permeability compared to empty vector tumors when both cell lines were inoculated with Matrigel (**Figure 2**). Representative 3D reconstructed maps of vascular volume (**Figure 2A, C**)and permeability (**Figures 2B , D**) show an increase of permeability but not vascular volume as shown in quantitated data in **Figures 2E, F**. The paracrine effect of VEGF on tumor cell growth is well established [8]. In addition to laminin, heparin sulphate proteoglycan, and collagen type IV, VEGF, is also present in Matrigel. Matrigel components, including VEGF, clearly increased vascular permeability in tumors derived from HIF silenced cells compared to tumors derived from empty vector cells. These data suggest that ECM components may modulate molecular targeting and highlight the importance of the tumor microenvironment in modifying HIF silencing effects. We are currently analyzing the effects on the metastatic burden in these systems.

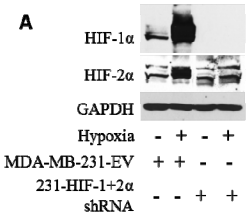


Figure 1A: Immunoblot showing expression of HIF-1α and HIF-2α in the genetically modified MDA-MB-231 cells. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein was used as a loading control.

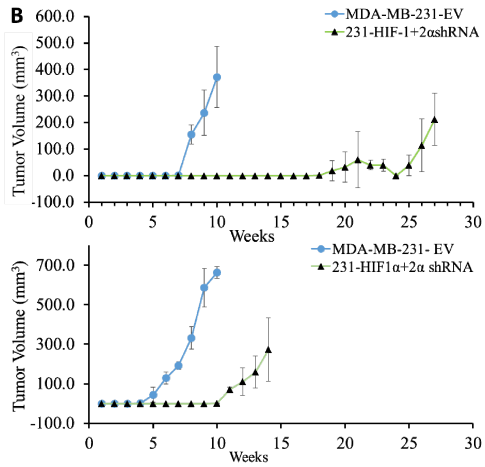


Figure 1B: Growth curves of tumors derived from MDA-MB-231-EV and 231-HIF-1+2α silenced cells. The upper panel displays growth curves obtained in the absence of Matrigel (n=5), the lower panel displays growth curves in the presence of Matrigel (n=3). Values are Mean ± S.E.M.

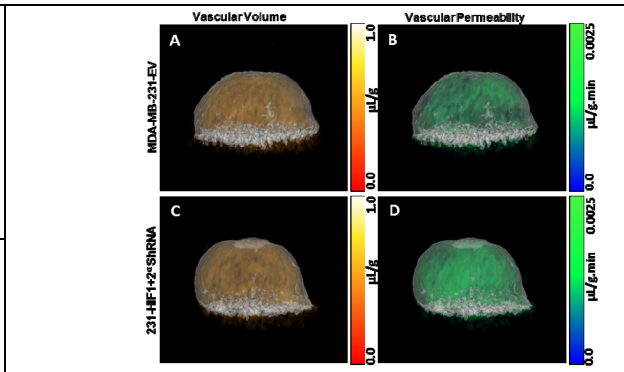
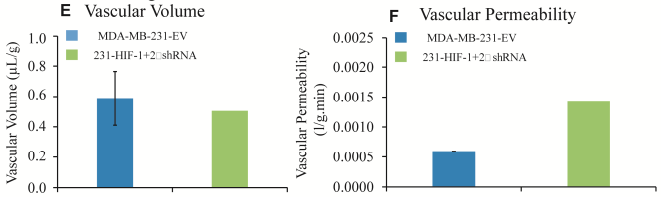


Figure 2: Three-dimensional images reconstructed from the qDCE-MRI data. (A, C) Orange color represents 3D calculated vascular volume (µl/g) maps and (B, D) green color represents vascular permeability (µl/(g.min) maps. Bar plot shows mean vascular volume (E) and mean vascular permeability (F). Error bars represent S.D.



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