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Hypoxia Inducible Factors Modify Collagen I Fibers in MDA-MB-231 Triple Negative Breast Cancer Xenografts[1,2]

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Abstract

Hypoxia inducible factors (HIFs) are transcription factors that mediate the response of cells to hypoxia. HIFs have wide-ranging effects on metabolism, the tumor microenvironment (TME) and the extracellular matrix (ECM). Here we investigated the silencing effects of two of the three known isoforms, HIF-1α and HIF-2α, on collagen 1 (Col1) fibers, which form a major component of the ECM of tumors. Using a loss-of-function approach for HIF-1α or 2α or both HIF-1α and 2α, we identified a relationship between HIFs and Col1 fibers in MDA-MB-231 tumors. Tumors derived from MDA-MB-231 cells with HIF-1α or 2α or both HIF-1α and 2α silenced contained higher percent fiber volume and lower inter-fiber distance compared to tumors derived from empty vector MDA-MB-231 cells. Depending upon the type of silencing, we observed changes in Col1 degrading enzymes, and enzymes involved in Col1 synthesis and deposition. Additionally, a reduction in lysyl oxidase protein expression in HIF-down-regulated tumors suggests that more non-cross-linked fibers were present. Collectively these results identify the role of HIFs in modifying the ECM and the TME and provide new insights into the effects of hypoxia on the tumor ECM.

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Introduction

Hypoxia and the stabilization of hypoxia inducible factors (HIFs) that mediate the adaptive response of cancer cells to hypoxia have been associated with increased invasiveness and metastasis [1]. Three known HIF isoforms, HIF-1α, HIF-2α and HIF-3α with separate as well as overlapping roles have been identified [2–4]. Of these, HIF-1α is the most widely investigated. Triple negative breast cancer (TNBC) is an aggressive form of breast cancer that occurs in 15–20% of breast cancer patients. TNBCs relapse, display refractory drug-resistance, and metastasize earlier than other subtypes [5,6]. In TNBCs, overexpression of HIF-1α was associated with poor outcome in early stage disease [7]. In a recent study, immunization against HIF-1α inhibited the growth of basal mammary tumors [8]. The same study showed elevation of HIF-1α-specific IgG in TNBC patients.

Abbreviations: Col1, Collagen I; DAB, 3,3′-diaminobenzedine; DS, Double Silenced; ECM, Extracellular Matrix; HIF, Hypoxia Inducible Factor; H&E, Hematoxylin and eosin; LOX, Lysyl Oxidase; MMP, Matrix Metalloproteinase; MT-MMP, Membrane-Type Matrix Metalloproteinase; P4H, Prolyl Hydroxylase; qRT-PCR, Quantitative Real Time Polymerase Chain Reaction; TME, Tumor Microenvironment; TNBC, Triple Negative Breast Cancer; SHG, Second Harmonic Generation; SMA, Smooth Muscle Actin.

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Cancer cells invade and migrate through the ECM on their metastatic journey [9–11]. Hypoxia and HIFs can facilitate invasion and metastasis by regulation of degradative enzymes and remodeling of the ECM [12]. Collagen 1 (Col1) fibers are a major component of the tumor ECM [13]. Col1 fibers also play a role in tumor development and metastasis [13–15]. High Col1 fiber density has been associated with tumor aggressiveness [13–15]. In breast cancers, high Col1 fiber density has been associated with increased malignancy [14]. The COL1A1 and COL1A2 genes produce pro-α1(I) and pro-α2(I) chains of the type I procollagen [16]. Prolyl 4-hydroxylases (P4Hs) are enzymes that catalyze the hydroxylation of proline residues on the pre-pro-collagen chains to form 4-hydroxyproline [17]. The hydroxylation of proline is a crucial step in forming procollagen type I that eventually forms mature Col1 after cross-linking by lysyl oxidase (LOX) [18]. LOX and the LOX-like family are enzymes that catalyze the cross-linking of Col1 [19]. High LOX or LOX-like expression has been associated poor prognosis in breast cancer [20–23]. Additionally, LOX and LOX-L2 are up-regulated in highly metastatic breast cancer cells [24]. LOX expression was found to be induced by hypoxia and regulated by HIF-1 in triple negative breast cancer cells [25]. Recently, HIF response elements were identified in the human LOX gene promoter [26].

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that degrade ECM proteins. MMP-1 and MMP-14, specifically, degrade and remodel Col1 fibers [27]. MMP-1 has been found to promote tumor growth and metastasis particularly to the brain [28]. MMP-14, or MT1-MMP, is a member of the membrane-type MMP subfamily (MT-MMP). Whereas other MMPs are secreted, MT-MMPs are part of a subfamily expressed as active proteins on the surface of cells [29,30]. MT1-MMP has previously been linked to angiogenesis and invasion [31,32].

Here, for the first time, we investigated the relationship between down-regulation of HIF-1α, HIF-2α, or combined HIF-1α and HIF-2α, on Col1 fiber patterns detected with second harmonic generation (SHG) microscopy of MDA-MB-231 triple negative breast cancer xenografts. To identify the molecular mechanisms underlying the differences in Col1 fiber patterns, we assayed LOX, P4HA1, P4HA2, MMP-1 and MT1-MMP in these tumors. Since cancer associated fibroblasts (CAFs) are a major source of Col1 fibers in tumors, we determined the numbers of CAFs in these tumors [33,34]. Our data provide new insights into the role of HIFs in regulating the tumor ECM.

Materials and Methods

Cells and Cell Culture Conditions

Cloning and generation of MDA-MB-231 cells stably expressing shRNA against HIF-1α, HIF-2α and both HIF-1α and HIF-2α using lentiviral transduction were performed as previously described [35,36]. Cells were maintained in RPMI 1640 medium (Mediatech, Manassas, VA, USA) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO), and maintained at 37 °C in a CO_2 incubator.

Tumor Studies

MDA-MB-231 cells expressing shRNA against HIF-1α (231-1α), HIF2-α (231-2α), or double silenced (231-DS) with cells expressing shRNA against both HIF-1α and HIF2-α were established using lentiviral transduction as previously described [35,36]. Engineered cells (2×10^6) were orthotopically inoculated in the mammary fat pad of female SCID mice, and monitored for tumor growth. Tumors were excised (231-EV, n = 10; 231-1α, n = 9; 231-2α, n = 8; 231-DS, n = 7) and used for *ex vivo* imaging and molecular analysis once tumor volumes were ~300 to 450 mm^3. All animal handling was conducted in accordance with the regulations outlined by the Institutional Animal Care and Use Committee of Johns Hopkins University.

SHG Microscopy

Briefly, tumors were paraffin-embedded and 5 μm thick sections obtained for immunohistochemical analysis. Hematoxylin and eosin (H&E) stained tumor sections were analyzed with SHG microscopy using an Olympus Laser Scanning FV1000 MPE multiphoton microscope (Olympus Corp., Center Valley, PA, USA) with a 25Xw/1.05XLPLN MP lens. Excitation was achieved at 860 nm and the second harmonic signal was detected at a wavelength of 430 nm. Multiple fields of view (FOVs) were acquired from each tumor section with a FOV of 423.52 × 423.52 μm^2. FOVs were spaced randomly throughout the tumor slide, avoiding necrotic regions. At least 6 FOVs were analyzed per tumor.

Quantification of fiber parameters including percent fiber volume and inter-fiber distance was performed using an in-house fiber analysis software as previously described [37]. The software was written using MATLAB 7.4.0 (The MathWorks, Natick, MA, USA).

RNA Isolation and qRT-PCR

RNA was isolated following a standard protocol (Qiagen, Valencia, CA, USA), and cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Briefly, tissue was homogenized with RLT buffer and passed through a QIAshredder to obtain RNA.

Quantitative real-time PCR (qRT-PCR) was performed using IQ SYBR Green Supermix and gene specific primers in the iCycler RT-PCR detection system (Bio-Rad, Hercules, CA, USA); 1 μl of 1:10 diluted cDNA was used. The expression of each gene was calculated relative to the house keeping gene, hypoxanthine phosphoribosyltransferase-1 (HPRT-1). The fold change between 231-HIF silenced and 231-EV tumors was calculated by comparing the change in threshold cycle (ΔCT) values. Confidence intervals were calculated based on a previously described model [38].

Protein Isolation and Immunoblotting

Protein expression levels of COL1A1, MMP-1, MMP-14, and LOX were quantified by immunoblotting. Approximately 100 μg of the whole cell protein lysates from tumor tissue, along with a prestained, broad range standard molecular weight marker were resolved on a 7% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane overnight after which the membrane was removed, blocked with 5% nonfat milk for 2 h and further incubated overnight with antibody. Antibodies used were rabbit-polyclonal anti-COL1A1 antibody (1:1000; OriGene, Rockville, MD, USA), rabbit polyclonal anti-MMP-1 antibody (1:1000 dilution; Neo BioLab, Woburn, MA, USA), rabbit polyclonal anti-MMP-14 antibody (1:1000 dilution; Neo BioLab, Woburn, MA, USA), mouse monoclonal anti-LOX antibody (1:1000 dilution; GeneTex, Inc., Irvine, CA, USA), or rabbit polyclonal anti-LOX-L1 antibody (1:1000 dilution; Sigma-Aldrich Corp. St. Louis, MO, USA). Horseradish peroxidase-conjugated secondary antibodies were used