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Cell Death and Survival

Molecular Cancer Research

Procollagen Lysyl Hydroxylase 2 Is Essential for Hypoxia-Induced Breast Cancer Metastasis

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Abstract

Metastasis is the leading cause of death among patients who have breast cancer. Understanding the role of the extracellular matrix (ECM) in the metastatic process may lead to the development of improved therapies to treat patients with cancer. Intratumoral hypoxia, found in the majority of breast cancers, is associated with an increased risk of metastasis and mortality. We found that in hypoxic breast cancer cells, hypoxia-inducible factor 1 (HIF-1) activates transcription of the *PLOD1* and *PLOD2* genes encoding procollagen lysyl hydroxylases that are required for the biogenesis of collagen, which is a major constituent of the ECM. High *PLOD2* expression in breast cancer biopsies is associated with increased risk of mortality. We show that PLOD2 is critical for fibrillar collagen formation by breast cancer cells, increases tumor stiffness, and is required for metastasis to lymph nodes and lungs. *Mol Cancer Res; 11(5); 456–66. ©2013 AACR.*

Introduction

During breast cancer progression, increased cancer cell proliferation and oxygen consumption lead to significantly reduced oxygen availability as compared with normal breast tissue (1, 2). Intratumoral hypoxia is associated with increased risk of invasion, metastasis, treatment failure, and patient mortality (3). The ability of cancer cells to survive and adapt to hypoxia depends on hypoxia-inducible factor 1 (HIF-1) and HIF-2, which induce the expression of more than 1,000 target genes, many of which are involved in angiogenesis, glucose metabolism, cell survival, invasion, and metastasis (4). HIF-1 is a heterodimeric protein that is composed of an O_2-regulated HIF-1α subunit and a constitutively expressed HIF-1β subunit (5). In mouse models, decreasing HIF-1α expression impedes tumor growth, angiogenesis, and breast cancer metastasis (6–10). In human breast cancer biopsies, increased HIF-1α protein levels are associated with an increased risk of metastasis and mortality, which is independent of patient stage, estrogen receptor expression, or lymph node status (11–15). HIF-2α is also O_2 regulated, dimerizes with HIF-1β, and promotes breast cancer progression (16).

Mammographic breast tissue density is a risk factor for breast cancer and collagen is a major contributor to tissue density (17–19). Fibrotic breast cancers have the poorest prognosis and highest rate of recurrence (20). During breast cancer progression, collagen fibers increase in density, straighten, bundle, and align (21). Several groups have observed that tumor cells preferentially invade along aligned collagen fibers (21–23). Furthermore, the pattern and extent of collagen alignment has prognostic significance in breast cancer (24). Increases in collagen cross-linking promote extracellular matrix (ECM) stiffening, which enhances cell growth, survival, tension, integrin signaling, and focal adhesion formation (25–27).

Collagens are the major fibrous proteins in the ECM and the most abundant proteins in the human body, modulating cell behavior primarily through interactions with integrins. Collagen biogenesis is a complex process involving extensive posttranslational modifications, including lysine hydroxylation (28). Hydroxylation of procollagen lysine residues occurs intracellularly and generates specific sites for glycosylation (29). Once collagen is secreted, collagen cross-linking occurs on lysine and hydroxylysine residues by the lysyl oxidase (LOX) family of enzymes (30). Cross-links involving hydroxylysine residues are more stable than those derived from lysine residues and less susceptible to matrix metalloproteinase degradation (31, 32). Thus, lysyl hydroxylases and oxidases are the key mediators of collagen cross-linking.

Three lysyl hydroxylase genes (*PLOD1*, *PLOD2*, and *PLOD3*) encoding isoforms of procollagen-lysine, 2-oxoglutarate 5-dioxygenase have been characterized (33). PLOD2 specifically hydroxylates lysines in the telopeptide of procollagens, whereas PLOD1 is responsible for lysine hydroxylation in the α-helical or central domain; the

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substrate specificity of PLOD3 is unknown (34–37). Enzymatic collagen cross-linking by lysyl oxidases occurs by the specific oxidative deamination of telopeptide lysine or hydroxylysine residues (30). Telopeptide lysines form cross-links, which are commonly found in soft tissue such as the skin (38). Telopeptide hydroxylysine residues react to form cross-links found in stiffer tissue such as cartilage and bone. Accordingly, overaccumulation of collagen in fibrotic conditions is caused by overhydroxylation of the collagen telopeptides and an increased amount of pyridinoline cross-links formed by hydroxylated telopeptide lysines (39–41). Pyridinoline cross-links are more stable and resistant to degradation. Furthermore, PLOD2 is the only lysyl hydroxylase that causes changes to collagen cross-linking patterns (41). Interestingly, PLOD1-deficient mice have a normal life span suggesting that PLOD2 may compensate for losses in PLOD1 expression (42). Although the role of PLODs in cancer has not been determined, their significance is evident in other diseases. Lysine hydroxylation is impaired in Bruck Syndrome (36) and Ehlers–Danlos Syndrome type VIA (43), whereas it is increased in fibrotic diseases (39).

We have previously shown that inhibition of HIF activity in breast cancer cells by RNA interference or treatment with digoxin, a drug that blocks HIF-1α protein expression, decreases both primary tumor growth and metastasis to lungs and lymph nodes (9, 10, 44, 45). Several studies have shown the importance of hypoxia-induced and HIF-regulated expression of LOX, LOX-like 2 (LOXL2), and LOXL4 for metastasis (10, 27, 45–47). Because lysyl hydroxylases are an integral part of collagen cross-linking and act upstream of LOX, we investigated their role in breast cancer metastasis. We analyzed the role of HIFs in regulating PLOD1 and PLOD2 expression in hypoxic breast cancer cells and investigated the effects of hypoxia-induced PLOD2 expression on tumor ECM properties and breast cancer metastasis.

Materials and Methods

Cell lines and culture

MDA-MB-231 (48) and MDA-MB-435 (49) human breast cancer cells were obtained from the National Cancer Institute (NCI) PS-OC Network Bioresource Core Facility and maintained in Dulbecco's modified Eagle's medium containing 10% FBS and antibiotics at 37°C in a 5% CO_2, 95% air incubator (20% O_2). The cells tested negative for the presence of *Mycoplasma* using a PCR-based detection kit. The cell lines were authenticated by short tandem repeat profiling. Hypoxic cells were maintained at 37°C in a modular incubator chamber (Billups-Rothenberg) flushed with a gas mixture containing 1% O_2, 5% CO_2, and 94% N_2. HIF-1α-null mouse embryo fibroblasts (MEF) were described previously (50).

shRNA, lentiviruses, and transduction

Vectors encoding short hairpin RNA (shRNA) targeting HIF-1α or HIF-2α were previously described (9). pLKO.1-puro shRNA expression vectors targeting PLOD2 were purchased from Sigma-Aldrich. Lentiviruses were packaged in 293T cells by cotransfection with plasmid pCMV-dR8.91 and a plasmid-encoding vesicular stomatitis virus G protein, using Lipofectamine 2000 (Invitrogen). Culture supernatants containing the viral particles were collected 48 hours after transfection and filtered using a 0.45-μm membrane. MDA-MB-231 and MDA-MB-435 cells were transduced with viral supernatant supplemented with 8 μg/mL Polybrene (Sigma-Aldrich) and selected in media containing 0.6 μg/mL puromycin.

Immunoblot assays

Aliquots of whole-cell lysates prepared in NP-40 buffer (150 mmol/L NaCl, 1% NP-40, 50 mmol/L Tris–HCl, pH 8.0) were fractionated by 8% SDS-PAGE. Conditioned medium was collected from cells and concentrated with 30% ammonium sulfate overnight at 4°C followed by centrifugation at 30,000 × *g* for 1 hour. Antibodies against HIF-1α (BD Transduction Laboratory), PLOD1, PLOD2, HIF-2α, COL1A1 (Novus Biologicals), and β-actin (Santa Cruz) were used.

Orthotopic implantation and metastasis assays

Studies using 7- to 10-week-old female nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (NCI) were approved by the Johns Hopkins University Animal Care and Use Committee and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Mammary fat pad (MFP) injection, tumor growth measurements, and human genomic DNA extraction from mouse lungs were previously described (9).

Real-time quantitative reverse transcription PCR

RNA extraction and cDNA synthesis were conducted as previously described (9). The fold change in expression of each target mRNA relative to 18S rRNA was calculated on the basis of the threshold cycle (C_t) as $2^{-\Delta(\Delta C_t)}$, where $\Delta C_t = C_t$ (target) $- C_t$ (18S) and $\Delta(\Delta C_t) = \Delta C_t$ (20% O_2) $- \Delta C_t$ (1% O_2).

Fibrillar collagen staining

Tumor sections were stained with 0.1% picrosirius red (Direct Red 80, Sigma) and counterstained with Weigert's hematoxylin. To reveal fibrillar collagen, stained sections were imaged with an Olympus IX51 fluorescence microscope fitted with an analyzer (U-ANT) and polarizer (U-POT). The percentage fibrillar collagen was quantified by calculating the area of staining (by thresholding) relative to the total area of the section using MetaMorph analysis software.

Tumor collagen content measurements

Cells were harvested and hydrolyzed in 6 N HCl for 16 hours at 116°C overnight. Tumor tissue was excised, dried in a vacuum, and weighed followed by hydrolysis in 6 N HCl for 16 hours at 116°C. Hydroxyproline content was determined by a colorimetric method (51). Total protein was measured by the Bradford assay using a commercial kit (BioRad).

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Immunohistochemistry

Tumors, lungs, and lymph nodes were fixed in 10% formalin and paraffin embedded. Sections were dewaxed and hydrated. LSAB+ System (DAKO) was used for PLOD2, HIF-1α and vimentin staining according to the manufacturer's instructions. Inflated lung sections were stained with hematoxylin and eosin to detect metastatic foci as previously described (9, 10). 3,3′-Diaminobenzidine (DAB) and hematoxylin were deconvoluted using Image J and pseudocolored to assess colocalization.

Statistical analysis of microarray data

Gene expression from the Richardson breast cancer dataset (52) retrieved from the Oncomine database (http://www.oncomine.org) was analyzed as previously described (53). Unpaired Student *t* tests were conducted to compare *PLOD1* and *PLOD2* levels in normal versus breast cancer tissue using GraphPad Prism software. The breast carcinoma dataset (54), which included gene expression (expressed as a *z*-score) and clinical stage, was obtained from The Cancer Genome Atlas (http://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp). Prognostic significance of PLOD expression in patients with breast cancer was examined in the Pawitan microarray dataset (55). Survival plots and HR were created using Kaplan–Meier methods in GraphPad Prism Software.

Tumor stiffness measurements

A stepper motor (Harvard Apparatus) was used to impinge a 1-mm diameter probe perpendicular to a freshly excised and immobilized tumor, with the corresponding force measured using a FlexiForce Load/Force Sensor (Tekscan). A constant impingement rate was maintained and force was recorded at 300 Hz using ELF software (TekScan). The slope of the indentation depth (mm) versus impingement force (mN) was used as an effective stiffness measurement. Stiffness values corresponding to the tail of the distribution of slopes were discarded to avoid analyzing necrotic regions. For each tumor, 5 to 6 random locations were probed and mean values were calculated.

Results

PLOD1 and PLOD2 expression is HIF-1–dependent in hypoxic breast cancer cells

The expression of mRNAs encoding PLOD1 and PLOD2 is known to be induced by hypoxia in mouse fibroblasts (56). To determine if hypoxia induces the expression of procollagen lysyl hydroxylases in human breast cancer cells, we analyzed PLOD1, PLOD2, and PLOD3 mRNA levels in MDA-MB-231 cells following exposure to 20% or 1% O_2 for 24 hours. Expression of PLOD2 mRNA was induced greater than 4-fold by hypoxia, whereas PLOD1 mRNA expression was more modestly induced (approximately 2-fold), and PLOD3 mRNA expression was not induced by hypoxia. To determine if HIF-1α or HIF-2α was required for PLOD expression under hypoxic conditions, we generated MDA-MB-231 subclones that were stably transfected with an empty vector (shEV) or expression vector(s) encoding a shRNA targeted against HIF-1α (sh1α), HIF-2α (sh2α), or HIF-1α and HIF-2α (sh1/2α). Hypoxic induction of PLOD1 and PLOD2 mRNA (Fig. 1A) and protein (Fig. 1B) was blocked when expression of HIF-1α (but not HIF-2α) was abrogated by shRNA. PLOD1 and PLOD2 protein levels were increased within 12 hours and remained elevated for 72 hours of continuous hypoxia (Fig. 1C). In addition, MDA-MB-231 cells were treated with digoxin, which inhibits HIF-1α protein expression (57). Hypoxia-induced PLOD1 and PLOD2 mRNA expression was significantly decreased in digoxin-treated MDA-MB-231 cells (Fig. 1D). Hypoxia also induced PLOD1 and PLOD2 mRNA expression in wild-type, but not in HIF-1α-null, MEFs (Supplementary Fig. S1A).

PLOD expression is associated with human breast cancer progression

To investigate whether procollagen lysyl hydroxylase expression has clinical significance in breast cancer, we compared *PLOD1* and *PLOD2* gene expression in normal human breast and breast cancer tissue using the Oncomine database (www.oncomine.org). Analysis of a representative dataset (52) revealed that PLOD1 and especially PLOD2 mRNA expression levels were significantly greater in breast cancer tissue than in normal breast tissue (Fig. 2A). The results were similar when we interrogated the Cancer Genome Atlas (54) for *PLOD1* and *PLOD2* expression in breast cancer versus adjacent normal tissue (Fig. 2B). Kaplan–Meier curves of disease-specific survival stratified by PLOD1 or PLOD2 mRNA levels in a 159-breast cancer patient dataset (55) revealed that high PLOD2 expression (greater than the median value) was significantly associated with decreased disease-specific survival (Fig. 2C). Comparing patients whose tumors expressed both PLOD1 and PLOD2 mRNA at greater than median levels with patients whose expression levels were less than the median did not improve the survival prediction. These data indicate that *PLOD2* expression is specifically prognostic in breast cancer.

Decreased PLOD2 expression in breast cancer cells inhibits their invasiveness

Because PLOD2 is the only PLOD family member that is significantly upregulated in fibrosis (36) and *PLOD2* expression was informative for breast cancer prognosis, we investigated the role of PLOD2 in breast cancer invasion and metastasis. We generated MDA-MB-231 subclones that were stably transfected with an empty vector (shLKO.1) or a vector encoding either of 2 different shRNAs against PLOD2 (shPL2-1 and shPL2-2). Immunoblot assays confirmed loss of PLOD2 protein expression in the knockdown subclones (Fig. 3A). The proliferation of subclones transfected with shLKO.1, shPL2-1, or shPL2-2 was similar in tissue culture (Fig. 3B). When injected into the MFP of NOD/SCID mice, the growth rate (Fig. 3C) and final tumor

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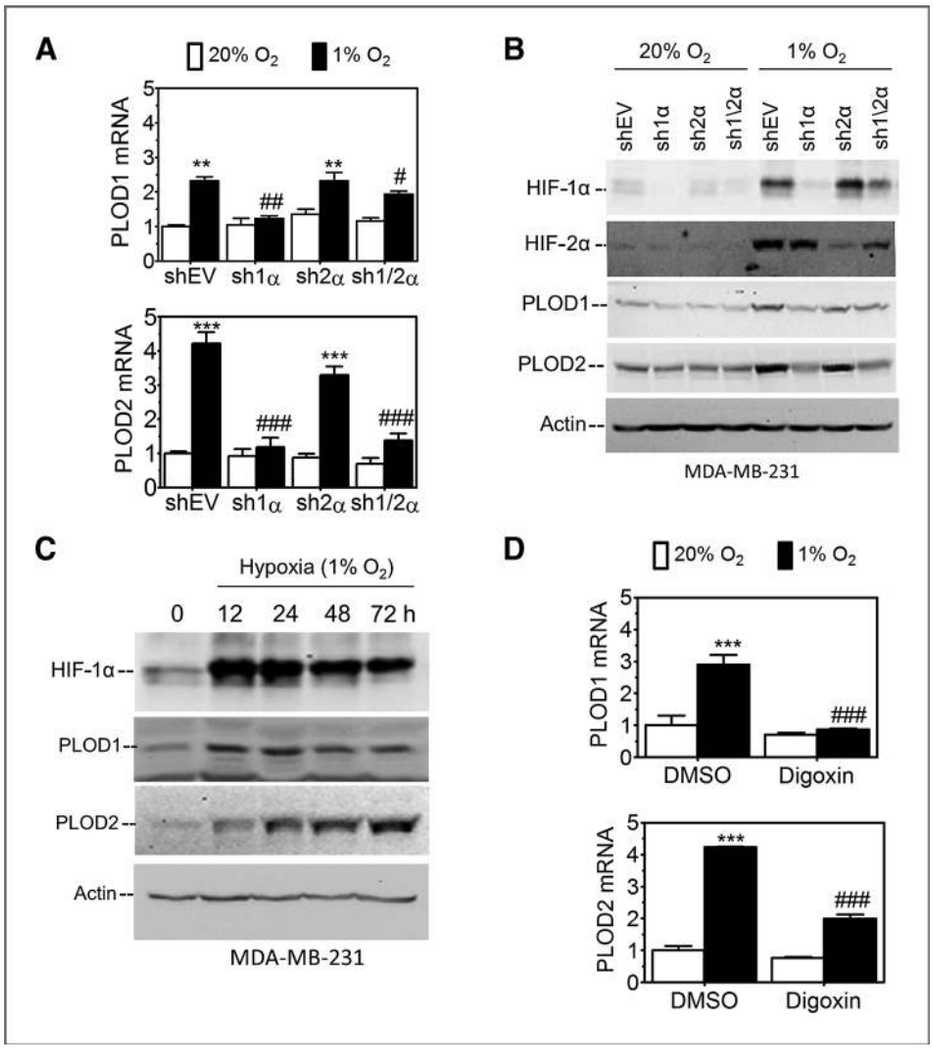

Figure 1. Knockdown of HIF-1α expression blocks PLOD1 and PLOD2 induction under hypoxic conditions. A, levels of PLOD1 and PLOD2 mRNA were analyzed by quantitative reverse transcription PCR (qRT-PCR) in MDA-MB-231 subclones, which were stably transfected with empty vector (shEV) or vector encoding HIF-1α shRNA (sh1α), HIF-2α shRNA (sh2α), or HIF-1α and HIF-2α shRNAs (sh1/2α), and exposed to 20% or 1% O_2 for 24 hours (mean ± SEM; $n = 3$); ***, $P < 0.001$; **, $P < 0.01$ versus shEV at 20% O_2; ###, $P < 0.001$; ##, $P < 0.01$; #, $P < 0.05$ versus shEV at 1% O_2 (one-way ANOVA with Bonferroni posttest). B, immunoblot assays were conducted using lysates prepared from MDA-MB-231 subclones exposed to 20% or 1% O_2 for 48 hours. C, immunoblot assays were conducted using lysates of parental MDA-MB-231 cells that were exposed to 1% O_2 for the indicated time in hours. D, PLOD1 and PLOD2 mRNA levels were analyzed by qRT-PCR in MDA-MB-231 cells exposed to vehicle [dimethyl sulfoxide (DMSO)] or 200 nmol/L digoxin at 20% or 1% O_2 for 24 hours. ***, $P < 0.001$ versus DMSO-treated 20% O_2; ###, $P < 0.001$ versus DMSO-treated 1% O_2 (one-way ANOVA with Bonferroni posttest).

weight (Fig. 3D) were similar among subclones. We confirmed that PLOD2 knockdown was maintained throughout the experiment (Fig. 3E). Immunohistochemical staining of control and knockdown tumors revealed intense PLOD2 staining in perinecrotic (hypoxic) regions of the control shLKO.1 tumors, which was markedly reduced in PLOD2-knockdown tumors (Fig. 3F). HIF-1α and PLOD2 staining colocalized within the perinecrotic region of control tumors (Fig. 3G). Control tumors showed extensive evidence of invasion into adjacent fat and muscle tissue, whereas PLOD2-knockdown tumors maintained a distinct tumor–stroma boundary (Fig. 3H). Taken together, these data show that *PLOD2* expression does not affect tumor growth but promotes local tissue invasion.

Decreased PLOD2 expression in breast cancer cells inhibits metastasis

Primary tumors derived from the injection of MDA-MB-231 cells into the MFP of NOD/SCID mice spontaneously metastasize to the lungs. The overall metastatic burden in the lungs was quantified by isolating genomic DNA from the mouse lung followed by real-time quantitative PCR (qPCR) using primers that only amplify human DNA (Fig. 4A). Lung metastasis was also assessed histologically by hematoxylin and eosin staining (Fig. 4B). The percentage of lung area occupied by breast cancer cells (Fig. 4C) and the number of metastatic foci in each lung section (Fig. 4D) confirmed the qPCR results (Fig. 4A).

We also assessed breast cancer cell infiltration of the ipsilateral axillary lymph nodes in control and PLOD2-knockdown tumor-bearing mice by immunohistochemical staining of human vimentin. The lymph nodes of mice that received MFP injection of shLKO.1 control cells were enlarged and completely infiltrated with breast cancer cells, whereas the follicular lymph node structure and size were maintained in the PLOD2-knockdown tumor-bearing mice (Fig. 4E). Using imaging software, quantitative analysis of lymph nodes was conducted by separating the staining signals into a vimentin signal (Fig. 4F, left) and a hematoxylin signal (Fig. 4F, middle). The area of vimentin staining

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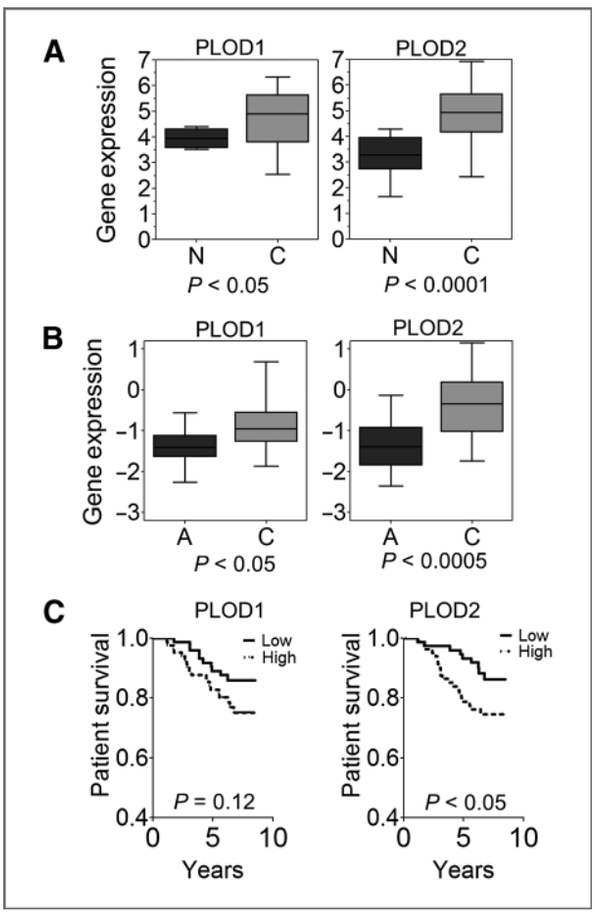

Figure 2. PLOD1 and PLOD2 expression in patients with breast cancer. A and B, *PLOD1* and *PLOD2* mRNA levels (presented as normalized microarray intensity values) in normal breast ($n = 7$) and breast cancer ($n = 40$) tissues (A) or in paired adjacent normal and breast cancer tissues from The Cancer Genome Atlas ($n = 28$; B) are shown. The box represents the 25th through 75th percentiles and whiskers represent the minimum and maximum range of the data. *P* values were determined by Student's *t* test. C, Kaplan–Meier analysis of disease-specific survival of 159 patients with breast cancer stratified by PLOD1 (left) or PLOD2 (right) mRNA expression in the primary tumor above the median level (High) or below the median level (Low).

within the lymph node above a background threshold was captured (Fig. 4F, right) and quantified (Fig. 4G). Lymph node infiltration by PLOD2-deficient breast cancer cells was reduced by greater than 4-fold (Fig. 4G). Taken together, the data presented in Fig. 4 show that PLOD2 expression is essential for breast cancer metastasis to lung and lymph nodes.

PLOD2 knockdown prevents fibrillar collagen formation in breast tumors

To determine if PLOD2 knockdown inhibits collagen secretion, we analyzed the levels of collagen 1A1 in conditioned media from MDA-MB-231 subclones following 72 hours of culture. Collagen secretion by MDA-MB-231 cells was not affected by PLOD2 knockdown (Fig. 5A). We used the Ehrlich method (58) to assess overall collagen content in control and PLOD2-knockdown tumors. The procedure involves the hydrolysis of tumor tissue and subsequent determination of hydroxyproline content. Given that approximately 12.5% of collagen consists of hydroxyproline, the amount of collagen in the tissue can be readily determined. Using this method, we determined that the overall collagen content in primary tumors was 8% of the total protein. PLOD2-knockdown did not affect overall collagen deposition in tumors (Fig. 5B). Because PLOD2 is important for collagen cross-linking, which is required for the formation of collagen fibers, we next analyzed fibrillar collagen content in control and PLOD2- knockdown tumor sections, using picrosirius red staining. Cross-linked collagen fibrils stained with picrosirius red can be detected when viewed under circularly polarized light. Collagen fibers were concentrated and aligned in the perinecrotic (hypoxic) regions of control tumors and absent in PLOD2-deficient tumors (Fig. 5C). Using image analysis to threshold the collagen fibers and compare the area occupied by collagen fibers with the total area of tumor in the section revealed a greater than 50% reduction in collagen fiber formation (Fig. 5D), which was associated with a corresponding decrease in tumor stiffness (Fig. 5E), in PLOD2-deficient tumors. Staining of serial sections revealed colocalization of fibrillar collagen (picrosirius red staining) and PLOD2 (immunohistochemistry) in perinecrotic regions (Fig. 5F), which is consistent with the biochemical function of PLOD2 in promoting fibrillar collagen formation. Taken together, the results shown in Fig. 5 show that PLOD2 does not affect collagen deposition but is required for collagen fibers to form within tumors.

PLOD2 activity is required for metastasis of MDA-MB-435 breast cancer cells

The results presented in Figs. 1–5 show a critical role for PLOD2 expression in local tissue invasion and metastasis of MDA-MB-231 cells to the axillary lymph node and lungs without affecting primary tumor growth. To show that these effects of PLOD2 are a general feature of metastatic breast cancer cells, we analyzed MDA-MB-435 cells (49). We generated MDA-MB-435 subclones stably transfected with an empty vector (shLKO.1) or a vector expressing either of 2 shRNAs against PLOD2 (shPL2-1 or shPL2-2; Fig. 6A and Supplementary Fig. S2) and orthotopically injected them into the MFP of NOD/SCID mice. Primary tumor growth was unaffected by the knockdown of PLOD2 (Fig. 6B), whereas local invasion of breast cancer cells was evident in control tumors only (Fig. 6C). Lung metastasis was significantly impaired by PLOD2 knockdown (Fig. 6D–G). Human vimentin staining of mouse lymph nodes (Fig. 6H) revealed an 80% reduction in lymph node infiltration by PLOD2-deficient breast tumors (Fig. 6I). Finally, decreasing PLOD2 in MDA-MB-435 cells reduced tumor stiffness (Fig. 6J). Taken together, the results presented in Figs. 1–6 show that hypoxia induces PLOD2 expression in 2 different metastatic breast cancer cell lines with similar effects on tumor stiffness, local invasion, and metastasis to lungs and lymph nodes.

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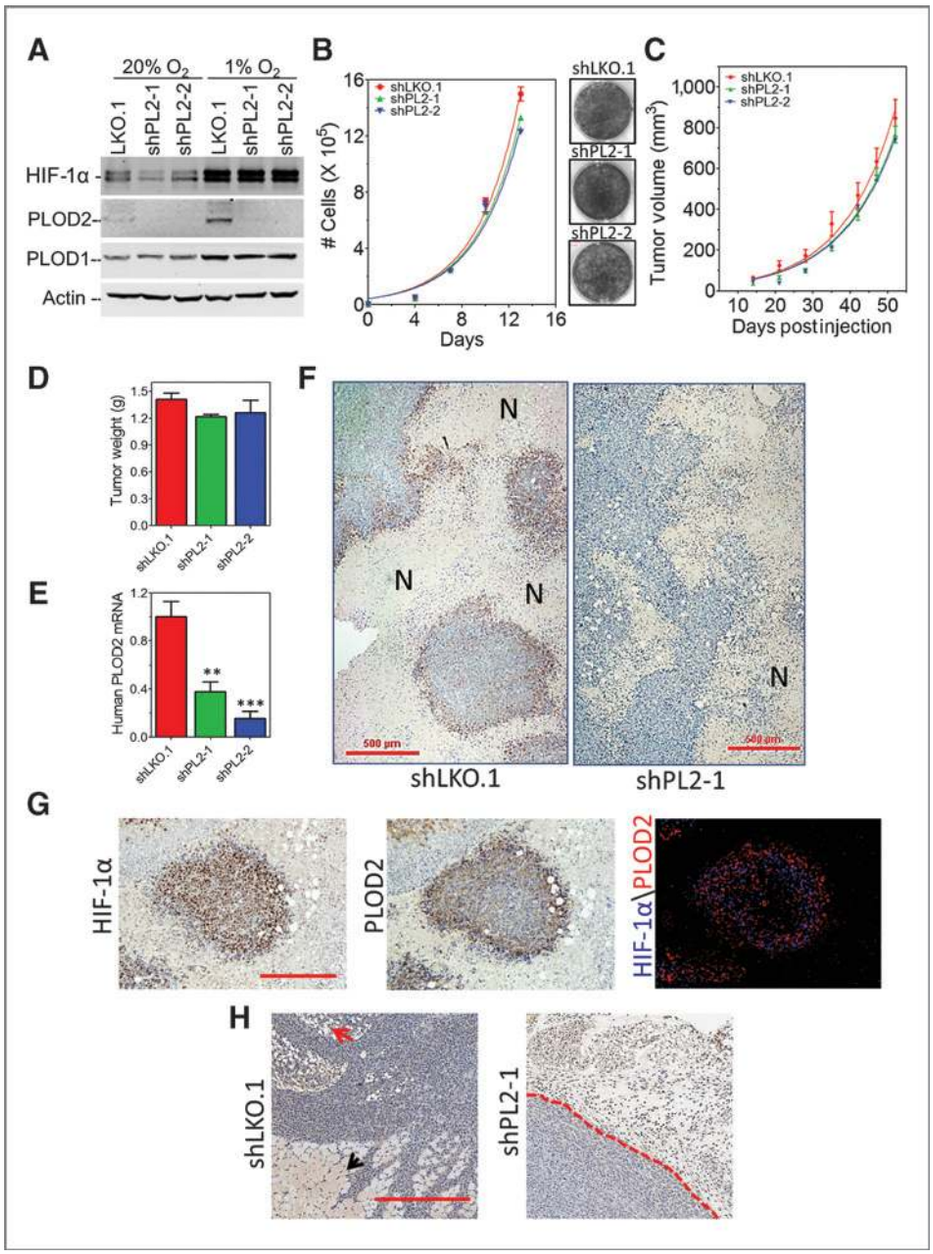

Figure 3. PLOD2 knockdown in MDA-MB-231 cells inhibits local tissue invasion. A, immunoblot assays were conducted using lysates prepared from MDA-MB-231 control (shLKO.1) or PLOD2-knockdown (shPL2-1 and shPL2-2) subclones exposed to 20% or 1% O_2 for 48 hours. B, proliferation of subclones was determined by Trypan blue staining on the indicated days. Crystal violet staining of tissue culture dishes on day 12 is shown (right). C–E, the subclones were injected into the MFP of NOD/SCID mice and tumor volume was plotted versus time (C), final tumor weight (g) was determined (D), and human PLOD2 mRNA levels were determined by qRT-PCR (E; mean ± SEM; $n = 5$; one-way ANOVA with Bonferroni posttest; ***, $P < 0.001$; **, $P < 0.01$ versus shLKO.1). F, immunohistochemical staining of primary tumor sections for PLOD2 is shown. Scale bar, 500 μm. G, immunohistochemical staining of primary tumor sections for HIF-1α and PLOD2 is shown. Images were deconvoluted and pseudocolored to assess colocalization (right). Scale bar, 500 μm. H, hematoxylin staining of primary tumor sections. Invasion of shLKO.1 into adipose tissue (red arrow) or muscle (black arrow) is shown in the left panel. The boundary between shPL2 cells and normal tissue is indicated by dashed line in the right panel. Scale bar, 500 μm.

Discussion

HIF-1 regulates multiple steps in collagen biogenesis

Multiple sequential steps are required for collagen fibril formation including synthesis of procollagen polypeptides, hydroxylation of proline and lysine residues, triple helix formation, glycosylation, secretion to the extracellular space, cleavage of propeptides, deposition, and cross-linking of collagen molecules (fiber formation). Several studies have highlighted the role of hypoxia and HIF-1 in inducing the expression of multiple members of the LOX family of enzymes, which cross-link collagen that has been secreted into the ECM and promote breast cancer metastasis (45, 46). The present study shows that HIF-1 also promotes the initiating step of collagen cross-linking, which involves hydroxylation of collagen lysine residues, by activating *PLOD1* and *PLOD2* gene expression in hypoxic breast cancer cells (Fig. 7).

Collagen cross-linking is sufficient to promote metastasis

Recent evidence shows that LOX induces increased breast cancer stiffness by promoting collagen cross-linking, which facilitates integrin clustering to reduce tumor latency (27). Tumor-secreted LOX also has effects on collagen cross-linking in the lungs, which promotes premetastatic niche formation (47). Because LOX affects ECM both in the

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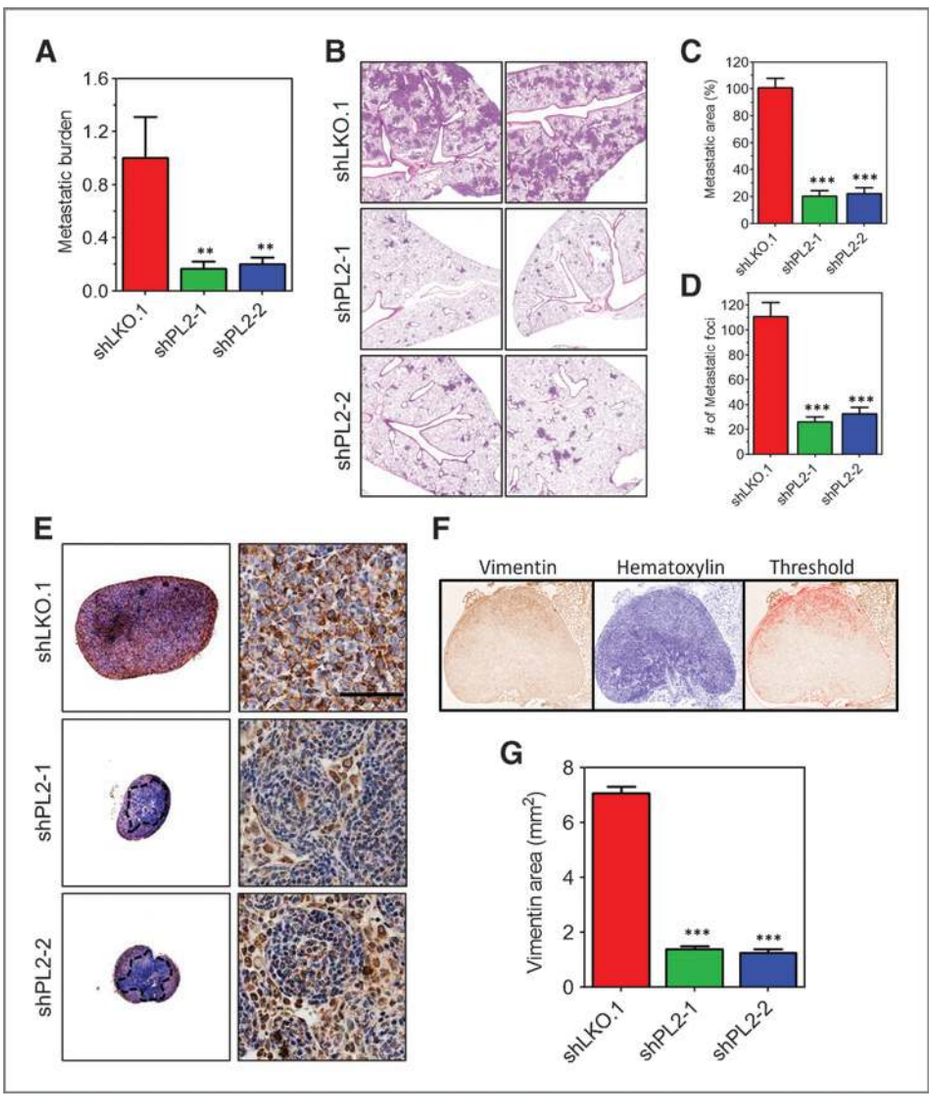

Figure 4. PLOD2 is essential for lymph node and lung metastasis. A, human genomic DNA content in lungs of tumor-bearing mice was quantified using qPCR with human-specific *HK2* gene primers (mean ± SEM; $n = 5$; one-way ANOVA). B, lung sections (5 mm × 5 mm) were stained with hematoxylin and eosin. C, metastatic area was determined by image analysis of 5 mm × 5 mm lung sections (mean ± SEM; $n = 5$; one-way ANOVA). D, the number of metastatic foci per 5 mm × 5 mm tumor section was determined (mean ± SEM; $n = 5$; one-way ANOVA). E, axillary lymph node sections were subjected to immunohistochemistry using an antibody specific for human vimentin. Staining of whole lymph nodes in 3.5 mm × 3.5 mm sections with preserved lymph node structure outlined in dashed line on left. High-power field of lymph node follicles is shown on right. Scale bar, 100 μm. F and G, vimentin staining (F) was quantified by image analysis (G; mean ± SEM; $n = 5$; one-way ANOVA). Bonferroni posttests were conducted for all ANOVAs. **, $P < 0.01$; ***, $P < 0.001$ versus shLKO.1.

primary tumor and at the metastatic site, it is difficult to discern whether increased collagen cross-linking in the tumor itself or within the lung parenchyma is necessary to promote metastasis. In contrast, PLOD2 is an intracellular enzyme that only promotes metastasis by cross-linking within the primary tumor. Our results show that inhibition of PLOD2 expression prevents collagen fiber formation in tumors and provides the first evidence that alterations in the fibrillar structure of collagen caused by changes in cross-linking specifically within a primary breast tumor promotes lung and lymph node metastasis.

The role of PLOD2 in establishing a tumor ECM that facilitates metastasis

PLOD2 likely influences metastasis by several mechanisms. First, fibrillar collagen content affects the biophysical properties of the ECM, thereby promoting invasion (27). In our study, PLOD2 knockdown reduced overall tumor stiffness and fibrillar collagen formation without affecting collagen deposition, leading to impaired local invasion into the surrounding tissue and decreased lymphatic and hematogenous metastasis to axillary lymph nodes and lungs, respectively. Second, collagen fiber alignment directs the motility of cancer cells *in vivo* (22). We found aligned collagen fibers were localized to perinecrotic (hypoxic) regions of tumors where the highest levels of PLOD2 expression were detected. Third, tissue fibrosis influences tumor progression by regulating soluble growth factor availability, distribution, and presentation to cells (59).

PLOD2 expression and breast cancer prognosis

We found that high levels of PLOD2 mRNA in the primary tumor are significantly associated with increased risk of mortality in women with breast cancer. Although before our work PLOD2 had not been studied intensively

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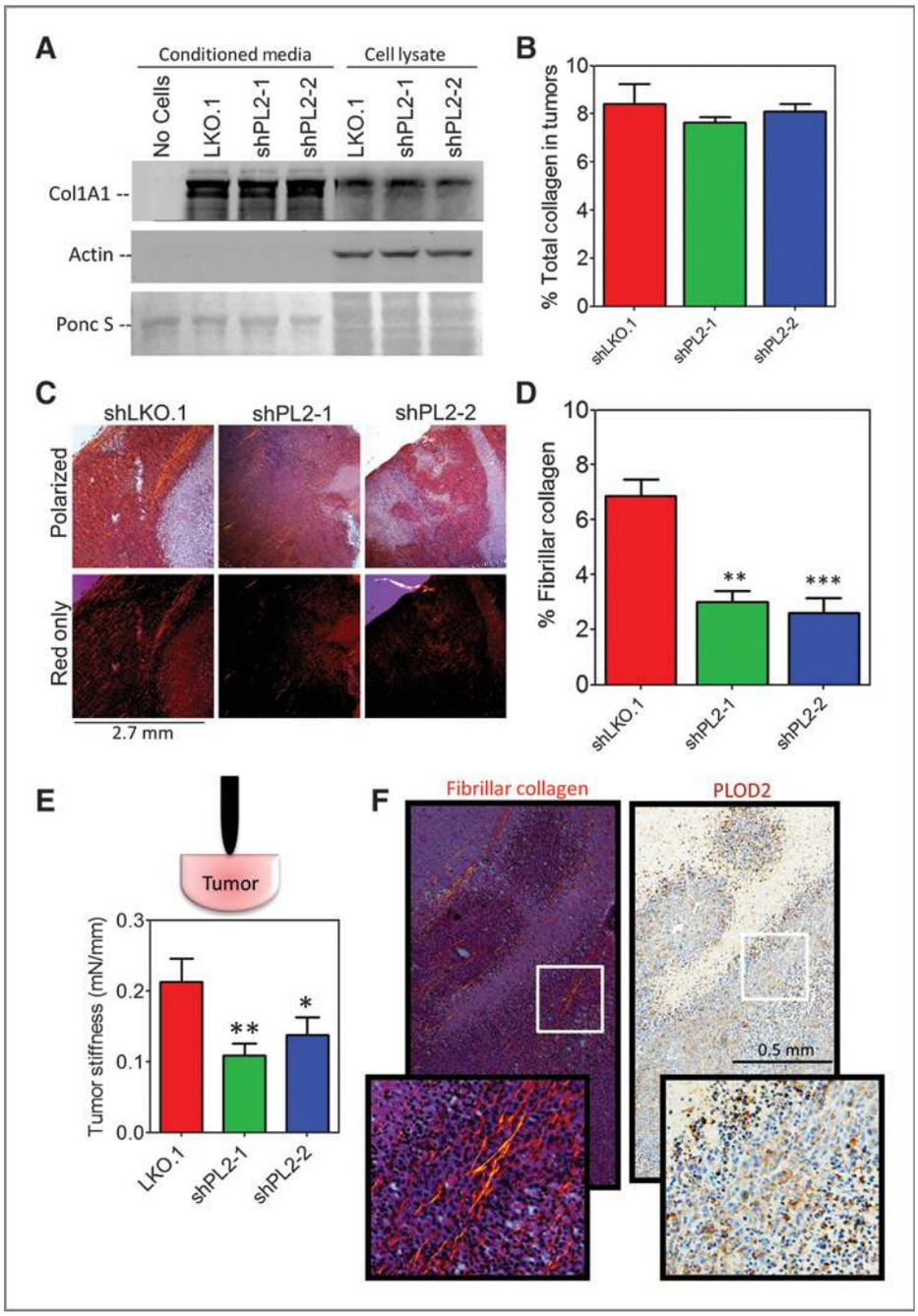

Figure 5. Knockdown of PLOD2 expression decreases fibrillar collagen formation but not total collagen deposition. A, collagen 1A1 protein levels in conditioned media and cell lysate of MDA-MB-231 subclones exposed to 1% O_2 for 72 hours were determined by immunoblot assay. Actin was used as a loading control for cell lysate. Ponceau S staining was used as a loading control for conditioned media. B, collagen content of tumors was determined using the collagen hydroxyproline assay. C, tumor sections from the indicated subclones were stained with picrosirius red and imaged under circularly polarized light (top) and collagen fiber staining above a background threshold was captured and subjected to image analysis (bottom). D, picrosirius red staining of 3 sections from 5 mice per group was quantified by image analysis to determine the area of the tumor section occupied by collagen fibers (% fibrillar collagen; mean ± SEM, one-way ANOVA). E, the stiffness of freshly dissected control (shLKO.1) or shPLOD2 tumors was determined (mean ± SEM, one-way ANOVA). F, picrosirius red staining to detect cross-linked collagen fibers (left) and immunohistochemical staining to detect PLOD2 (right) were conducted on serial control tumor sections. Bonferroni posttest was conducted for all ANOVAs. *, $P<0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus shLKO.1 in (D) and (E).

in the context of tumor biology, a survey of the literature suggests that it may be relevant for cancer prognosis. In several microarray studies, *PLOD2* was identified in gene signatures associated with cervical cancer, glioblastoma, hepatocellular carcinoma, and gastric cancer (60–63). Furthermore, *PLOD2* was 1 of 17 genes implicated as a potential mediator of breast cancer metastasis to the brain (64). Interestingly, in agreement with the results of our study, neither PLOD1 nor PLOD3 was implicated in these studies. These data indicate that our findings in a mouse model of breast cancer are likely to have clinical significance.

PLOD2 and breast cancer treatment

Fibrotic breast cancers have the poorest prognosis and highest rate of recurrence (20). An evaluation of tumor biopsies from 196 patients with breast cancer identified a histologic signature characterized by bundles of straightened and aligned collagen fibers that predicted adverse patient outcome, which was independent of tumor grade

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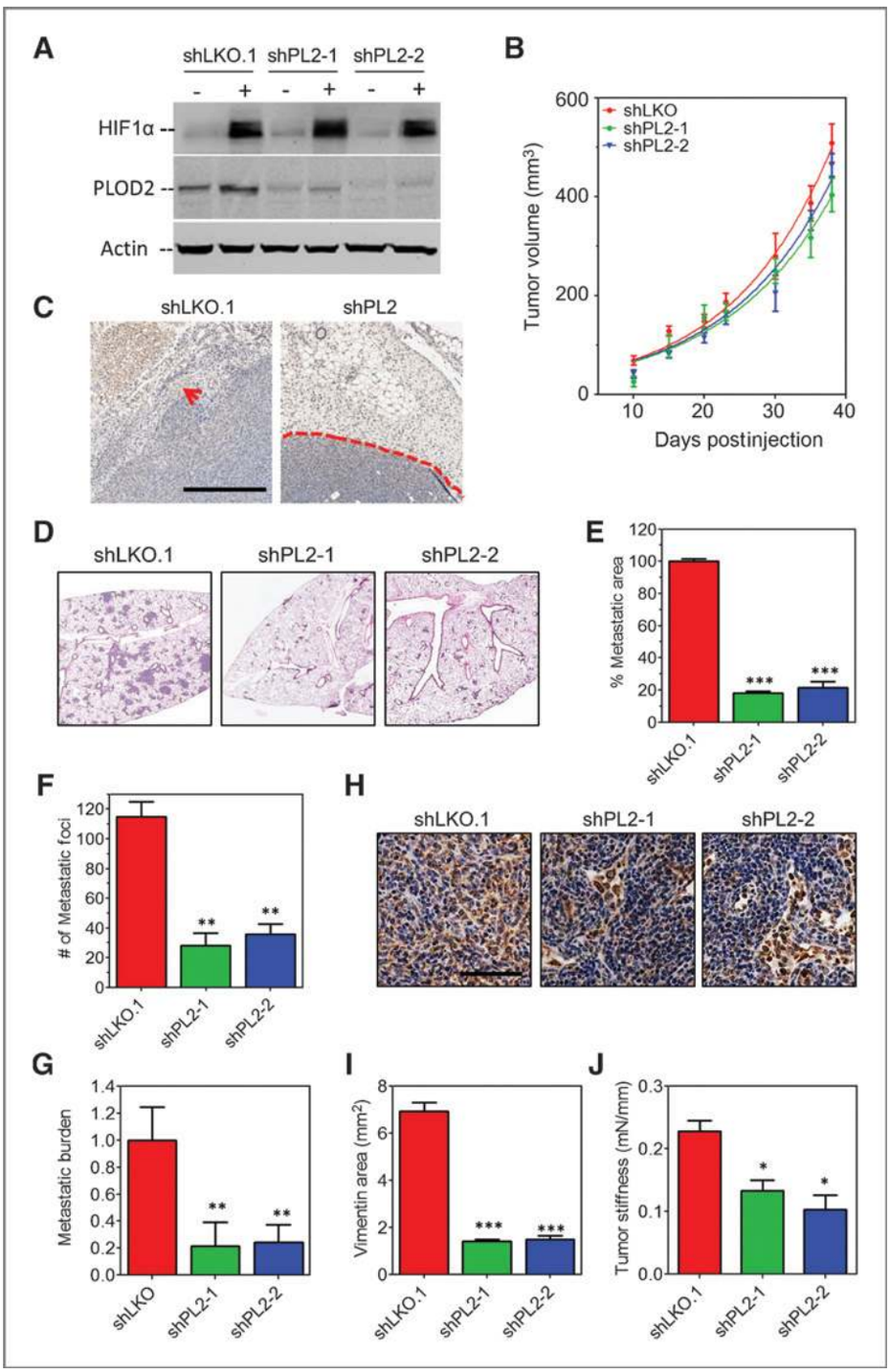

Figure 6. PLOD2 is essential for invasion and metastasis of MDA-MB-435 cells. A, immunoblot assays were conducted using lysates prepared from control (shLKO.1) or PLOD2-knockdown (shPL2-1 and shPL2-2) MDA-MB-435 subclones exposed to 20% or 1% O_2 for 48 hours. B, the indicated subclones were injected into the MFP of NOD/SCID mice and tumor volume was plotted versus time. C, hematoxylin staining of primary tumor sections. Invasion of shLKO.1 cells into adipose tissue (red arrow) is shown in the left panel. The well-defined boundary between shPL2 cells and normal tissue is indicated by the dashed line in the right panel. Scale bar, 0.5 mm. D, lung sections (5 mm × 5 mm) were stained with hematoxylin and eosin. E, metastatic area was determined by image analysis (mean ± SEM; $n = 5$; one-way ANOVA). F, the number of metastatic foci per 5 mm × 5 mm tumor section was determined (mean ± SEM; $n = 5$; one-way ANOVA). G, human genomic DNA content in mouse lungs was quantified using qPCR with human-specific *HK2* gene primers (mean ± SEM; $n = 5$; one-way ANOVA). H, ipsilateral axillary lymph node sections were subjected to immunohistochemistry using an antibody specific for human vimentin. Scale bar, 100 μm. I, vimentin staining was quantified by image analysis (mean ± SEM; $n = 5$; one-way ANOVA). J, the stiffness of freshly dissected control or shPLOD2 tumors was determined. Bonferroni posttests were conducted for all ANOVAs. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ versus shLKO.1.

or size, estrogen or progesterone receptor status, $HER2^{neu}$ status, lymph node status, or tumor subtype (24). This finding, taken together with the dramatic effect of PLOD2 knockdown on fibrillar collagen formation, indicates that procollagen lysyl hydroxylases represent a potential therapeutic target for the prevention of breast cancer metastasis. Drugs such as digoxin that inhibit HIF-1 activity may be beneficial because they reduce tumor fibrosis by blocking multiple steps in collagen biogenesis (Fig. 7) as well as impairing other critical steps in the metastatic process (9, 10, 44, 45). Furthermore, specific inhibitors of HIF-1 or procollagen lysyl hydroxylase activity may block

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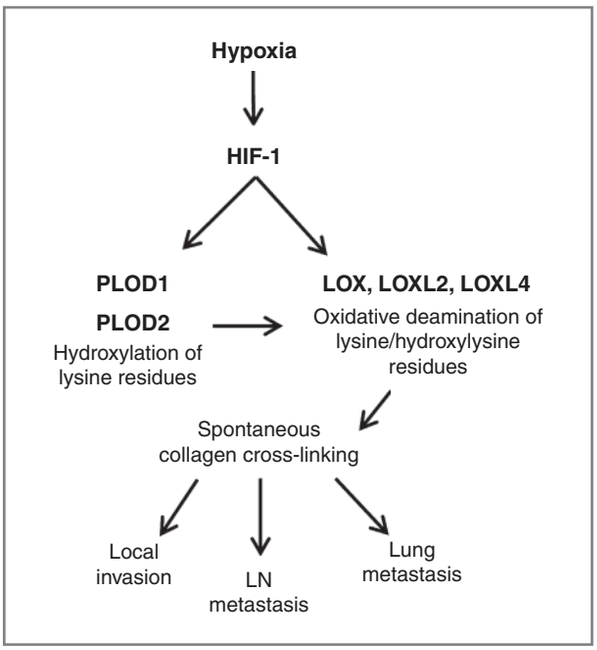

Figure 7. Hypoxia-induced and HIF-1–dependent expression of PLOD2 and LOX family members mediate cross-linking of collagen fibers, leading to increased invasion and metastasis of breast cancer cells.

collagen fiber biogenesis by both cancer cells and cancer-associated fibroblasts and their use can be guided by the analysis of HIF-1α or PLOD2 expression in diagnostic cancer biopsies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: D. Gilkes, G.L. Semenza
Development of methodology: D. Gilkes, P. Chaturvedi, D. Wirtz
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Gilkes, S. Bajpai, M.E. Hubbi, D. Wirtz
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Gilkes, S. Bajpai, G.L. Semenza
Writing, review, and/or revision of the manuscript: D. Gilkes, M.E. Hubbi, G.L. Semenza
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.C.-L. Wong, M.E. Hubbi
Study supervision: G.L. Semenza

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