

Collagen Prolyl Hydroxylases Are Essential for Breast Cancer Metastasis

Daniele M. Gilkes^{1,2,4}, Pallavi Chaturvedi^{1,2}, Saumendra Bajpai^{4,5}, Carmen C. Wong^{1,2}, Hong Wei^{1,2}, Stephen Pitcairn^{1,2}, Maimon E. Hubbi^{1,2}, Denis Wirtz^{4,5}, and Gregg L. Semenza^{1,2,3,4}

Abstract

The presence of hypoxia and fibrosis within the primary tumor are two major risk factors for metastasis of human breast cancer. In this study, we demonstrate that hypoxia-inducible factor 1 activates the transcription of genes encoding collagen prolyl hydroxylases that are critical for collagen deposition by breast cancer cells. We show that expression of collagen prolyl hydroxylases promotes cancer cell alignment along collagen fibers, resulting in enhanced invasion and metastasis to lymph nodes and lungs. Finally, we establish the prognostic significance of collagen prolyl hydroxylase mRNA expression in human breast cancer biopsies and show that ethyl 3,4-dihydroxybenzoate, a prolyl hydroxylase inhibitor, decreases tumor fibrosis and metastasis in a mouse model of breast cancer. *Cancer Res*; 73(11); 3285–96. ©2013 AACR.

Introduction

The majority of human breast cancers develop regions of severe intratumoral hypoxia (1, 2), leading to the activation of the hypoxia-inducible factors HIF-1 and HIF-2, which mediate adaptive transcriptional responses to reduced O₂ availability as well as stimulating invasion and metastasis (3). The HIFs are heterodimeric proteins that consist of an O₂-regulated HIF-1 α or HIF-2 α subunit and a constitutively expressed HIF-1 β subunit (3, 4). Genetic manipulations decreasing HIF-1 α expression impede tumor growth, angiogenesis, and metastasis in animal models (5–9). Immunohistochemical analyses of primary breast cancer biopsies have revealed that high HIF-1 α expression is associated with significantly increased risks of treatment failure, disease relapse, distant metastasis, and patient mortality (10–14). Increased HIF-2 α expression is also associated with increased risk of recurrence and mortality (15).

Cancer progression is also associated with an increase in extracellular matrix (ECM) deposition and stiffening, which enhances cell growth, survival, integrin signaling, and focal adhesion formation (16–21). Using mouse models that recapitulate the histologic progression of human breast cancer, mammary tumors exhibited a localized increase in collagen deposition (22, 23). As tumor size increased, collagen fibers straightened, bundled, and aligned (24). Several groups observed tumor cells preferentially invading along aligned collagen fibers (24–26). However, studies to date have not determined an underlying molecular mechanism for the increase in collagen fiber formation during tumor progression. In this study, we tested the hypothesis that HIF-1 is a direct regulator of increased collagen deposition, which in turn promotes invasion to drive the metastasis of hypoxic breast cancer cells.

Collagen biogenesis requires collagen prolyl 4-hydroxylase (P4H) activity to catalyze collagen proline hydroxylation. Three isoforms of the P4HA subunit (P4HA1, P4HA2, and P4HA3) form A₂B₂ tetramers with P4HB resulting in P4H1, P4H2, and P4H3 holoenzymes, respectively (27, 28). Proper hydroxylation is required for folding newly synthesized procollagen polypeptide chains into stable triple helical molecules, a prerequisite for extracellular secretion (29, 30). Following procollagen secretion, peptidases remove N- and C-terminal propeptides, and lysyl oxidase (LOX) cross-links triple-helical molecules to form mature collagen fibers.

Many studies have focused on the role of LOX family members in extracellular collagen crosslinking and tumor progression (9, 18, 31) without considering the rate-limiting step of collagen deposition. In this study, we determined that hypoxia-induced collagen prolyl hydroxylase expression promotes collagen deposition, which enhances invasion, leading to lymph node and lung metastasis. Elevated P4HA1 and P4HA2 mRNA levels in human breast cancers predict patient

Authors' Affiliations: ¹Vascular Program, Institute for Cell Engineering; ²McKusick-Nathans Institute of Genetic Medicine; ³Departments of Pediatrics, Oncology, Medicine, Radiation Oncology, and Biological Chemistry, The Johns Hopkins University School of Medicine; ⁴Johns Hopkins Physical Sciences-Oncology Center; ⁵Department of Chemical and Biomolecular Engineering, The Johns Hopkins University, Baltimore, Maryland

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

This work is dedicated in memoriam of Stephen Pitcairn, who was striving to make a difference.

Corresponding Author: G.L. Semenza, Johns Hopkins University School of Medicine, 733 N. Broadway, Suite 671, Baltimore, MD 21205. Fax: 443-287-5618; E-mail: gsemenza@jhmi.edu

doi: 10.1158/0008-5472.CAN-12-3963

©2013 American Association for Cancer Research.

mortality. Finally, we show that treating tumor-bearing mice with ethyl 3,4-dihydroxybenzoate (DHB), a hydroxylase inhibitor, decreases breast cancer fibrosis and metastasis.

Materials and Methods

Cell lines and culture

MDA-MB-231 and MDA-MB-435 cells were obtained from the National Cancer Institute (NCI) PS-OC Network Bioresource Facility and maintained in Dulbecco's Modified Eagle Medium with 10% FBS and antibiotics in a 5% CO₂, 95% air incubator (20% O₂). The cells tested negative for mycoplasma using a PCR-based detection kit. The cell lines were authenticated by short tandem repeat profiling. Hypoxic cells were maintained in a modular incubator chamber flushed with a gas mixture containing 1% O₂, 5% CO₂, and 94% N₂.

shRNA, lentiviruses, and transduction

Vectors encoding short hairpin RNA (shRNA) targeting HIF-1 α and HIF-2 α and virus production methods were previously described (8). pLKO.1-puro vectors encoding shP4HA1 and shP4HA2 were purchased from Sigma-Aldrich. P4HA1 and P4HA2 lentiviral expression plasmids were generated by inserting cDNA for the coding sequence into a pENTR Gateway vector (Invitrogen) followed by recombination with the pLenti-CMV/TO-Zeo-DEST vector.

Immunoblot assays

Aliquots of whole-cell lysates prepared in NP-40 buffer were fractionated by 8% SDS-PAGE. Conditioned medium was concentrated by ammonium sulfate precipitation at 4°C overnight followed by centrifugation. Antibodies against HIF-1 α (BD Transduction Laboratory), P4HA1, P4HA2, HIF-2 α , 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 carried out according to protocols approved by the Johns Hopkins University Animal Care and Use Committee (Baltimore, MD). Mammary fat pad (MFP) injection, tumor growth measurement, and human genomic DNA extraction from mouse lungs were previously described (8). Mice were treated with DHB [in 5% ethanol and 95% normal saline (v/v)] at 40 mg/kg/d by an intraperitoneal injection.

Real-time reverse transcription quantitative PCR

RNA extraction and cDNA synthesis was carried out as previously described (8). The 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 $\Delta C_t = C_t(\text{target}) - C_t(18S)$. For primer sequences, see Supplementary Table S1.

Fibrillar collagen staining

Tumor sections were stained with 0.1% picosirius red (Direct Red 80, Sigma) and counterstained with Weigert hematoxylin to reveal fibrillar collagen. Sections were imaged with an Olympus IX51 fluorescence microscope fitted with an

analyzer (U-ANT) and polarizer (U-POT). The percentage of collagen was quantified by calculating the area of red staining relative to the total area of the tumor section using MetaMorph analysis software.

Hydroxyproline content measurements

Cells were harvested and hydrolyzed in 6N HCl for 16 hours at 116°C. Tumor tissue was excised, dried in a vacuum, and hydrolyzed in 6N HCl for 16 hours at 116°C. Hydroxyproline content was determined by a colorimetric method (32). The total protein content was measured by the Bradford assay using a commercial kit (Bio-Rad).

Immunohistochemistry

Lungs and lymph nodes were fixed in 10% formalin and paraffin embedded. Sections were dewaxed and hydrated. LSAB+ System HRP Kit (DAKO) was used for P4HA1, P4HA2, and vimentin staining. HIF-1 α staining was conducted as described previously (33). Inflated lung sections were stained with hematoxylin and eosin to visualize metastatic foci.

Statistical analysis

All statistical analysis was carried out using GraphPad Prism software. Bonferroni posttests were conducted for all ANOVAs. Data from The Cancer Genome Atlas was obtained from <http://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp>. OncoPrint data was obtained from the Richardson microarray dataset (34). Survival analysis data was obtained from the Pawitan microarray dataset (35) accessible through the National Center for Biotechnology Information GEO database.

Tumor stiffness measurements

Tumor stiffness measurements were conducted using a stepper motor (Harvard Apparatus) with 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 versus impingement force was used as an effective stiffness measurement.

Results

Inhibition of HIF-1 α expression blocks hypoxia-induced P4HA1 and P4HA2 expression

The ECM is a critical factor in the tumor microenvironment (36–38). Analysis of MDA-MB-231 breast cancer cells exposed to 20% or 1% O₂ revealed that P4HA1 and P4HA2 mRNA were highly induced under hypoxia (Supplementary Fig. S1A). P4HA1 and P4HA2 protein levels were increased within 12 hours and remained elevated for 72 hours of continuous hypoxia (Supplementary Fig. S1B). To determine whether HIF-1 α or HIF-2 α was required for P4H expression under hypoxic conditions, we used MDA-MB-231 subclones stably transfected with an empty vector (EV) or expression vector(s) encoding shRNA targeting HIF-1 α (sh1 α), HIF-2 α (sh2 α), or HIF-1 α , and HIF-2 α (sh1/2 α ; ref. 8). Hypoxic induction of P4HA1 and P4HA2 mRNA (Fig. 1A) and protein (Fig. 1B)

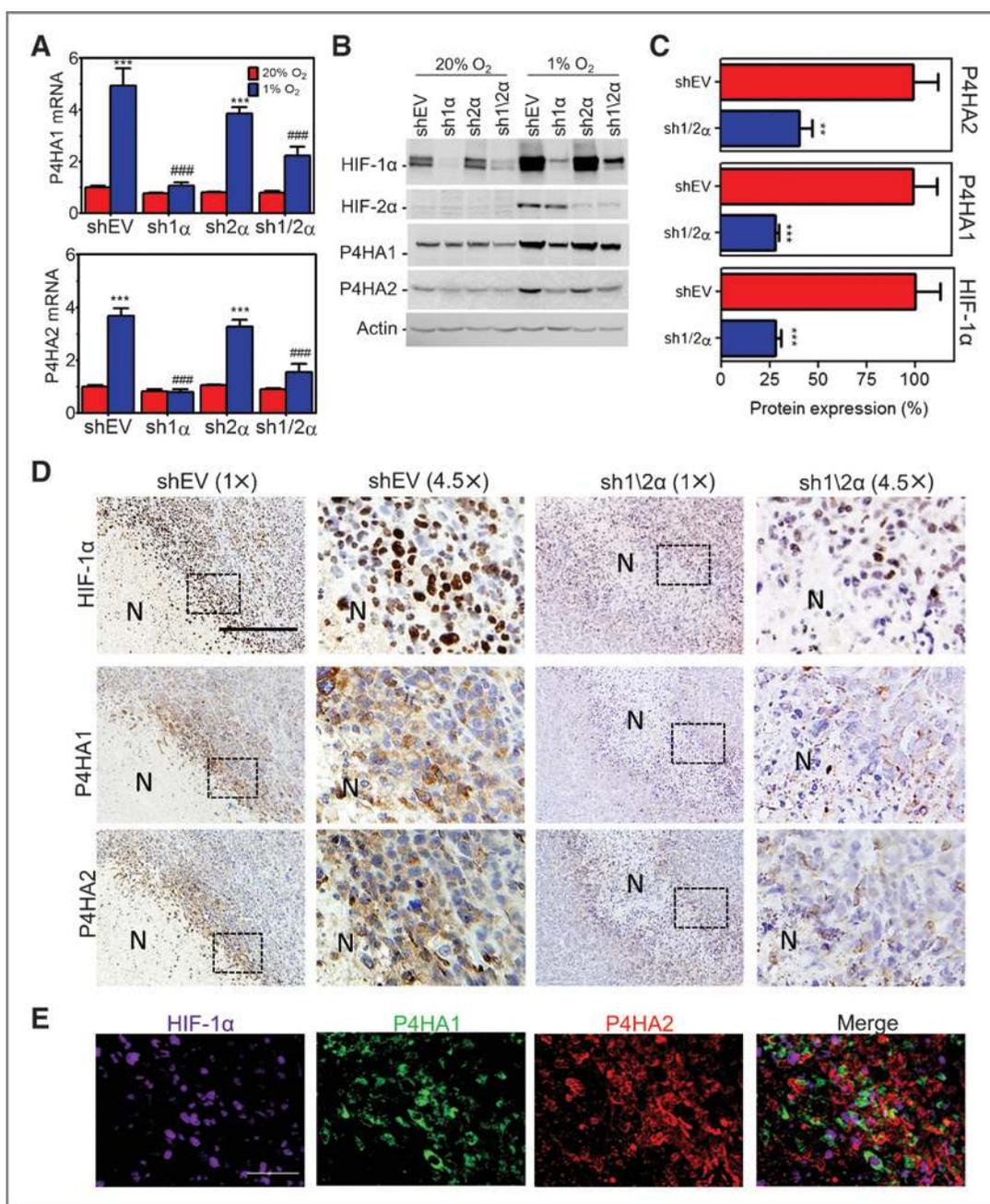


Figure 1. Knockdown of HIF-1 α blocks P4HA1 and P4HA2 induction under hypoxic conditions. **A**, expression of P4HA mRNAs was analyzed by qRT-PCR in MDA-MB-231 subclones exposed to 20% or 1% O₂ for 24 hours (mean \pm SEM; $n = 3$; ANOVA); ***, $P < 0.001$ vs. shEV (20% O₂); ###, $P < 0.001$ vs. shEV (1% O₂). **B**, immunoblot assays were carried out using lysates from MDA-MB-231 subclones exposed to 20% or 1% O₂ for 48 hours. **C**, immunoblot assays (Supplementary Fig. S1D) of tumor lysate were quantified by optical density and normalized to MDA-MB-231-shEV expression (mean \pm SEM; $n = 5$; Student t test). **, $P < 0.01$; ***, $P < 0.001$ vs. shEV. **D**, immunohistochemical staining of serial sections from shEV and sh1/2 α tumors. Scale bar, 150 μ m. N, necrotic region. **E**, shEV images (D) were deconvoluted and pseudocolored to assess colocalization. Scale bar, 25 μ m.

expression was blocked when HIF-1 α (but not HIF-2 α) was silenced. Hypoxia-induced P4HA1 and P4HA2 mRNA expression was also blocked by treatment with digoxin (Supplementary Fig. S1A), which inhibits HIF-1 α expression (39). P4HA1 and P4HA2 expression was also induced under hypoxic conditions in MCF-7 breast cancer cells and

MCF10A immortalized breast epithelial cells (Supplementary Fig. S1C).

To assess hypoxic induction of collagen hydroxylases *in vivo*, we injected MDA-MB-231-shEV and -sh1/2 α subclones into the MFP of NOD/SCID mice. P4HA protein levels were significantly decreased in tumors derived from sh1/2 α

as compared with shEV cells (Fig. 1C and Supplementary Fig. S1D). Immunohistochemistry of tumor sections showed intense nuclear HIF-1 α staining in perinecrotic (hypoxic) regions of the shEV tumors (Fig. 1D), which colocalized with P4HA1 and P4HA2 (Fig. 1D and E and Supplementary Fig. S1E). Staining of HIF-1 α , P4HA1, and P4HA2 was attenuated in sh1/2 α tumor sections. Residual expression of HIF-1 α in sh1/2 α tumors may reflect incomplete silencing of HIF-1 α expression in cancer cells or host-derived stromal cells, which were not subjected to silencing, or both. Taken together, these data show that HIF-1 promotes collagen prolyl hydroxylase expression in hypoxic breast cancer cells both *in vitro* and *in vivo*.

Collagen prolyl hydroxylase knockdown inhibits breast cancer growth and metastasis

To determine whether prolyl hydroxylases are required for metastasis, we generated MDA-MB-231 subclones stably transfected with an empty vector (shLKO.1) or vector encoding either of 2 different shRNAs against P4HA1 (shHA1-1 and shHA1-2) or P4HA2 (shHA2-1 and shHA2-2). Immunoblot assays confirmed the knockdown of P4HA1 and P4HA2 (Fig. 2A). Primary tumor growth was attenuated by knockdown of either P4HA1 or P4HA2 (Fig. 2B and C). Differences in cell proliferation were observed *in vitro* (Supplementary Fig. S2A) but were enhanced *in vivo* where the ECM may play a more important role. More strikingly, spontaneous lung metastasis

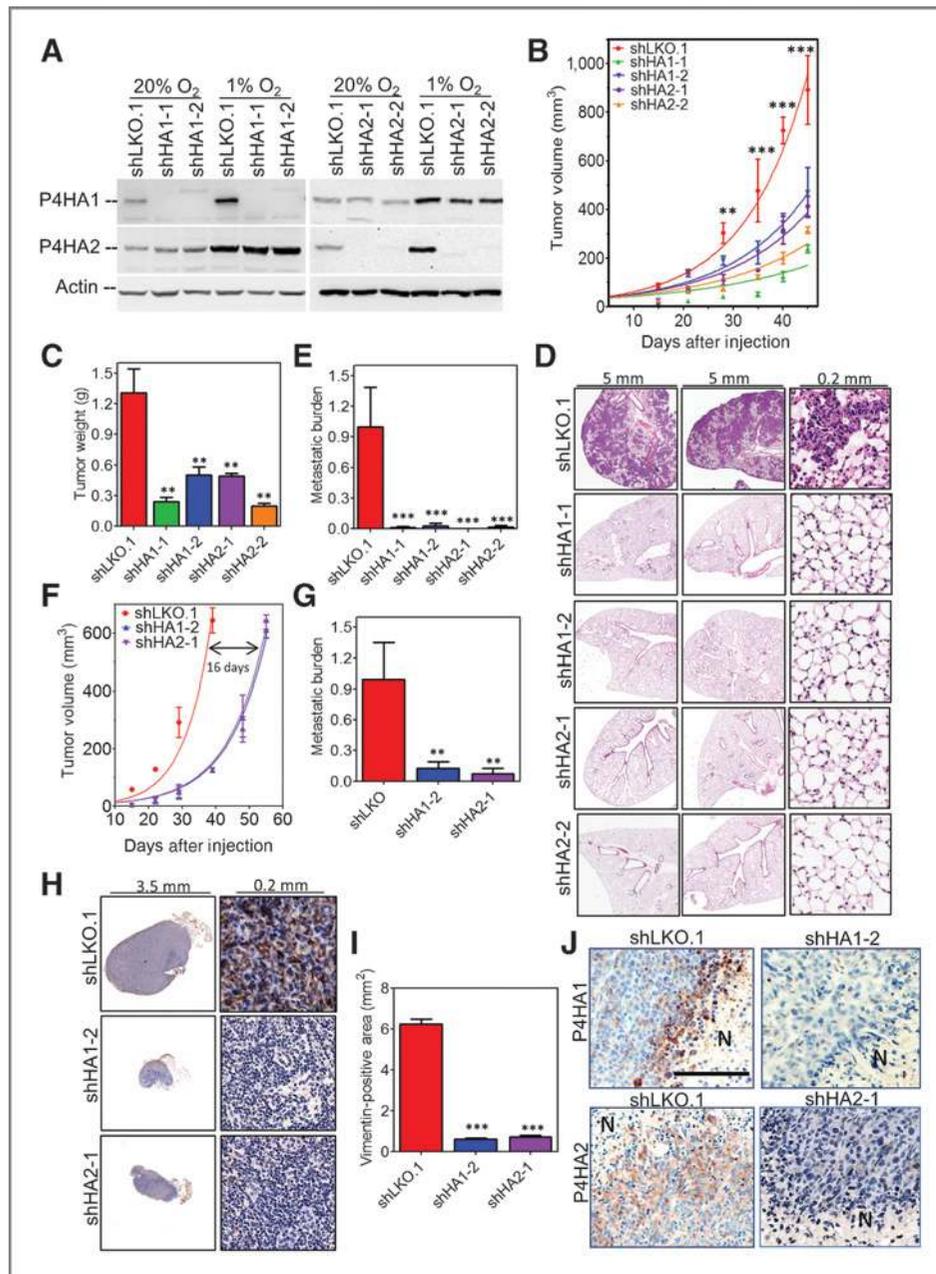


Figure 2. Knockdown of P4HA1 or P4HA2 expression in MDA-MB-231 cells inhibits tumor growth and metastasis. A, immunoblot assays were conducted using lysates prepared from MDA-MB-231 subclones exposed to 20% or 1% O₂ for 48 hours. B, tumor volume measured versus time (mean \pm SEM; $n = 5$; ANOVA). C, final tumor weight (in grams; mean \pm SEM; $n = 5$; ANOVA). D, lung sections [5 \times 5 mm (left) or 0.2 \times 0.2 mm (right)] were stained with hematoxylin and eosin. E, human genomic DNA content in mouse lungs was quantified using qPCR (mean \pm SEM; $n = 5$; ANOVA). F, tumor volume versus time. G, human DNA content in mouse lungs determined by qPCR (mean \pm SEM; $n = 5$; ANOVA). H, immunohistochemical staining of axillary lymph node sections for human vimentin. I, vimentin staining quantified by image analysis (mean \pm SEM; $n = 5$; ANOVA). J, immunohistochemical staining of primary tumor sections for P4HA1 or P4HA2. Scale bar, 200 μ m. **, $P < 0.01$; ***, $P < 0.001$ vs. shLKO.1.

was completely abrogated in mice bearing tumors derived from P4HA1 or P4HA2 knockdown cells (Fig. 2D). Genomic DNA was isolated from contralateral lungs and quantitative real-time PCR (qPCR) using human-specific primers demonstrated that P4HA1 or P4HA2 knockdown reduced metastasis by more than 99% (Fig. 2E).

To take into account differences in primary tumor growth rates, we repeated the MFP injection and isolated tumors, lungs, and lymph nodes when primary tumor volumes reached 600 mm³. Control tumors reached 600 mm³ in 39 days, whereas P4HA knockdown tumors required 55 days to reach the same tumor volume and weight (Fig. 2F and Supplementary Fig. S2B). Despite the additional residence time for P4HA knockdown cells, no lung metastases were observed (Supplementary Fig. S2C). Human genomic DNA content in the lungs indicated a 90% reduction in metastasis of P4HA knockdown cells (Fig. 2G). In addition, lymphatic metastasis was assessed using human vimentin immunohistochemical staining of ipsilateral axillary lymph nodes (Fig. 2H). The area of vimentin staining was used to assess breast cancer cell infiltration of mouse lymph nodes. Depletion of either P4HA1 or P4HA2 reduced lymphatic metastasis over 5-fold (Fig. 2I). Furthermore, the follicular lymph node morphology was maintained only in the knockdown mice. Immunohistochemical staining for P4HA1 and P4HA2 confirmed expression in the perinecrotic regions of control (shLKO.1) tumors and markedly decreased expression in knockdown tumors (Fig. 2J). These results were consistent with markedly reduced human-specific P4HA1 and P4HA2 mRNA expression, whereas mouse P4HA1 and P4HA2 mRNA expression was not changed (Supplementary Fig. S2D–S2G). Thus, expression of P4HA1 and P4HA2 by breast cancer cells promotes primary tumor growth and lymphatic dissemination and is absolutely required for lung metastasis independent of tumor size.

To quantify the extent to which cancer cells contributed to collagen hydroxylase activity, we used primers specific for human, mouse, or human and mouse P4HA1 mRNA. In control (shLKO.1) tumors, human P4HA1 mRNA accounted for 87 ± 5% (mean ± SD) of total RNA, whereas mouse P4HA1 mRNA accounted for 9 ± 5% (Supplementary Table S2), suggesting the majority of P4HA1 activity originated from the MDA-MB-231 cells.

Collagen hydroxylase knockdown decreases collagen deposition *in vitro* and *in vivo*

The hydroxylation of collagen by prolyl hydroxylases is crucial for the folding, stability, and secretion of the collagen triple helix of all fibrillar collagens (30, 40). We found that secretion of type I collagen was abrogated by the knockdown of HIF-1 α , P4HA1, or P4HA2 (Fig. 3A and B). We examined the localization of collagen deposition in tumor sections using picrosirius red staining, which detects all fibrillar collagen (including types I, II, III, V, XI, XXIV, and XXVII) when viewed under circularly polarized light (21). Overall collagen content was greatest in perinecrotic regions (Fig. 3C and D), corresponding to sites of highest HIF-1 α and P4HA expression (Fig. 1D). Analysis of control tumors

revealed highly aligned collagen fibers in the perinecrotic region of tumors, whereas the bulk of the tumor contained discontinuous, fragmented collagen fibers (Fig. 3D). The knockdown of P4HA expression in breast cancer cells resulted in a marked decrease in tumor collagen content (both by picrosirius red and Masson trichrome staining) and tumor stiffness *in vivo* (Fig. 3E and F and S3C). We confirmed a reduction in hydroxylase activity by measuring collagen hydroxyproline content in tumor lysates, which correlated with reduced P4HA1/2 mRNA expression (Fig. 3G and S2H-I). Differences in collagen content were also observed when we compared tumor sections from control shEV and sh1/2 α subclones (Supplementary Fig. S3A and S3B), indicating that HIF-1-dependent expression of prolyl hydroxylases promotes collagen deposition in breast tumors. To confirm that our results were not cell line specific, we generated MDA-MB-435 subclones (Supplementary Fig. S4A). Primary tumor growth and metastasis were similar to the corresponding MDA-MB-231 subclones (Supplementary Fig. S4B). Lung metastasis was severely impaired by the P4HA knockdown and correlated with decreased tumor stiffness (Supplementary Fig. S4C–S4E).

Collagen hydroxylase expression promotes cancer cell invasion

Recent studies have shown that straightened and aligned collagen fibers in tumor samples are predictive of patient mortality (41), presumably because collagen fibers provide directional cues that dictate cell morphology and promote cell migration (24, 42) as well as induce stiffness, which promotes tissue tension to enhance cancer progression (16, 18). To determine whether collagen hydroxylase expression by breast cancer cells promotes invasion *in vivo*, we analyzed control and P4HA knockdown tumor margins. Knockdown tumors showed no evidence of invasion into adjacent adipose tissue, whereas all control tumors showed evidence of extensive local invasion (Fig. 4A).

Because *in vitro* assays of invasion (such as Boyden chamber assays) do not test the contribution of ECM to invasion, we generated cell-free three-dimensional matrices from confluent cultures of MDA-MB-231 control or P4HA knockdown cells. We seeded naïve MDA-MB-231 cells on these cell-derived matrices and analyzed cell morphology. Compared with cells plated on tissue culture plastic or ECM derived from P4HA knockdown cells, cells seeded on ECM derived from shLKO.1 cells had a spindle-shaped mesenchymal morphology (Fig. 4B), which is required for cell motility and invasion (42, 43). To determine whether collagen deposited within tumors promotes an invasive cell phenotype, MDA-MB-231 cells were labeled with CMFDA (5-chloromethylfluorescein diacetate) and seeded onto control or hydroxylase knockdown tumor sections. Following 24 hours of cell seeding, sections were stained with picrosirius red and imaged for cell–collagen interactions (Fig. 4C). In control tumors, with abundant collagen content, the MDA-MB-231 cells aligned themselves along collagen fibers with a spindle-shaped morphology, which was quantified by calculation of the elliptical factor (Fig. 4C, bar graph). In contrast, the P4HA

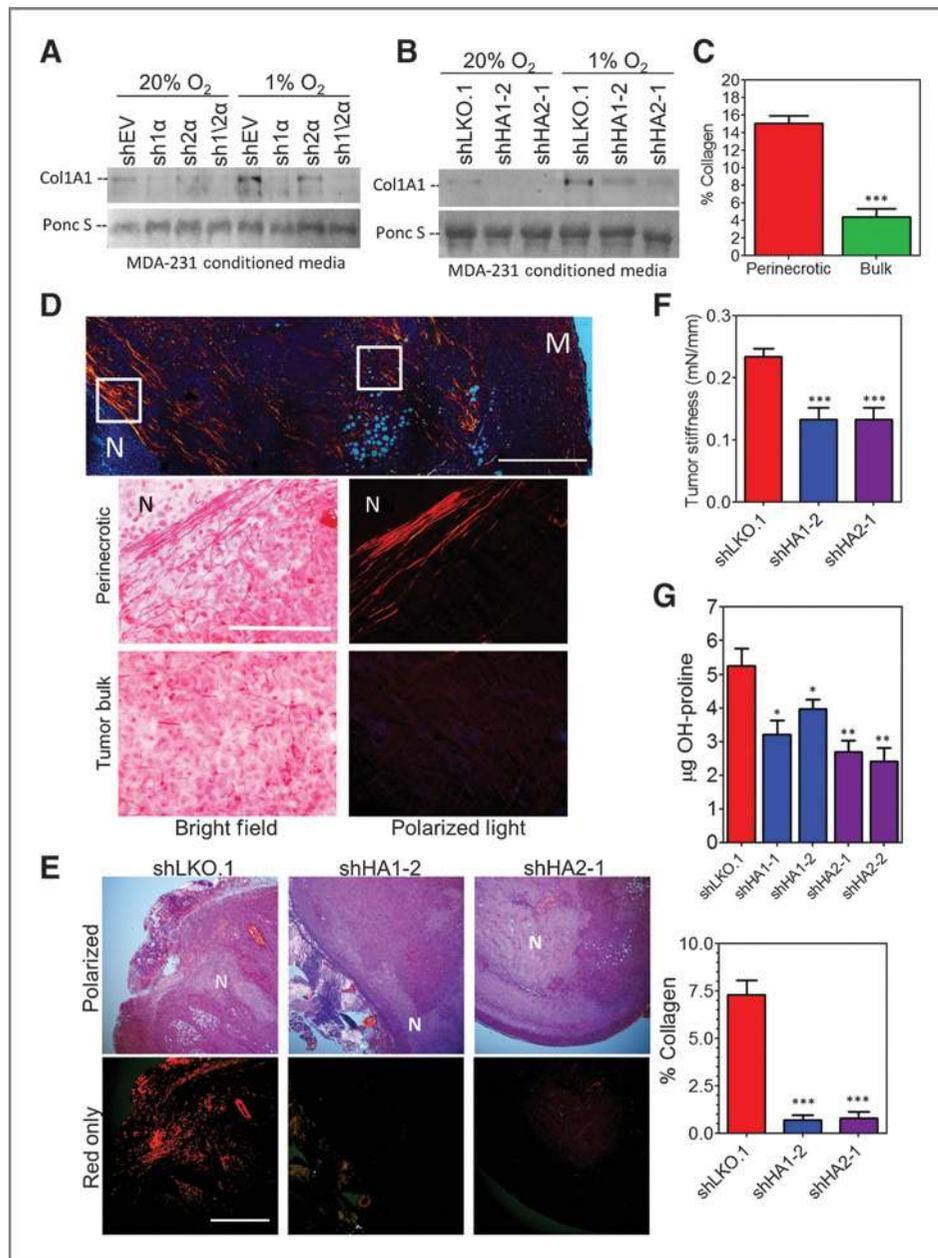


Figure 3. Collagen prolyl hydroxylase expression is associated with collagen content and tumor stiffness. A and B, immunoblot assay of collagen 1A1 from conditioned media of MDA-MB-231 subclones exposed to 20% or 1% O₂ for 72 hours. Ponceau S (Ponc S) staining was used as a loading control. C and D, picosirius red staining of control tumor sections imaged under circularly polarized light. The percentage of collagen in perinecrotic regions was compared with bulk tumor regions (white boxes) in C. Top scale bar, 1 mm; bottom scale bar, 0.1 mm; N, necrotic; M, tumor margin. E, picosirius red staining quantified by image analysis (mean ± SEM; *n* = 5; ANOVA). F, the stiffness of freshly dissected control, shP4HA1, or shP4HA2 tumors was determined (mean ± SEM; one-way ANOVA). G, the hydroxyproline content of tumor tissue was determined per mg of total protein (mean ± SEM; *n* = 5; ANOVA). *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001 vs. shLKO.1.

knockdown tumors did not contain sufficient collagen to promote cell elongation or directionality.

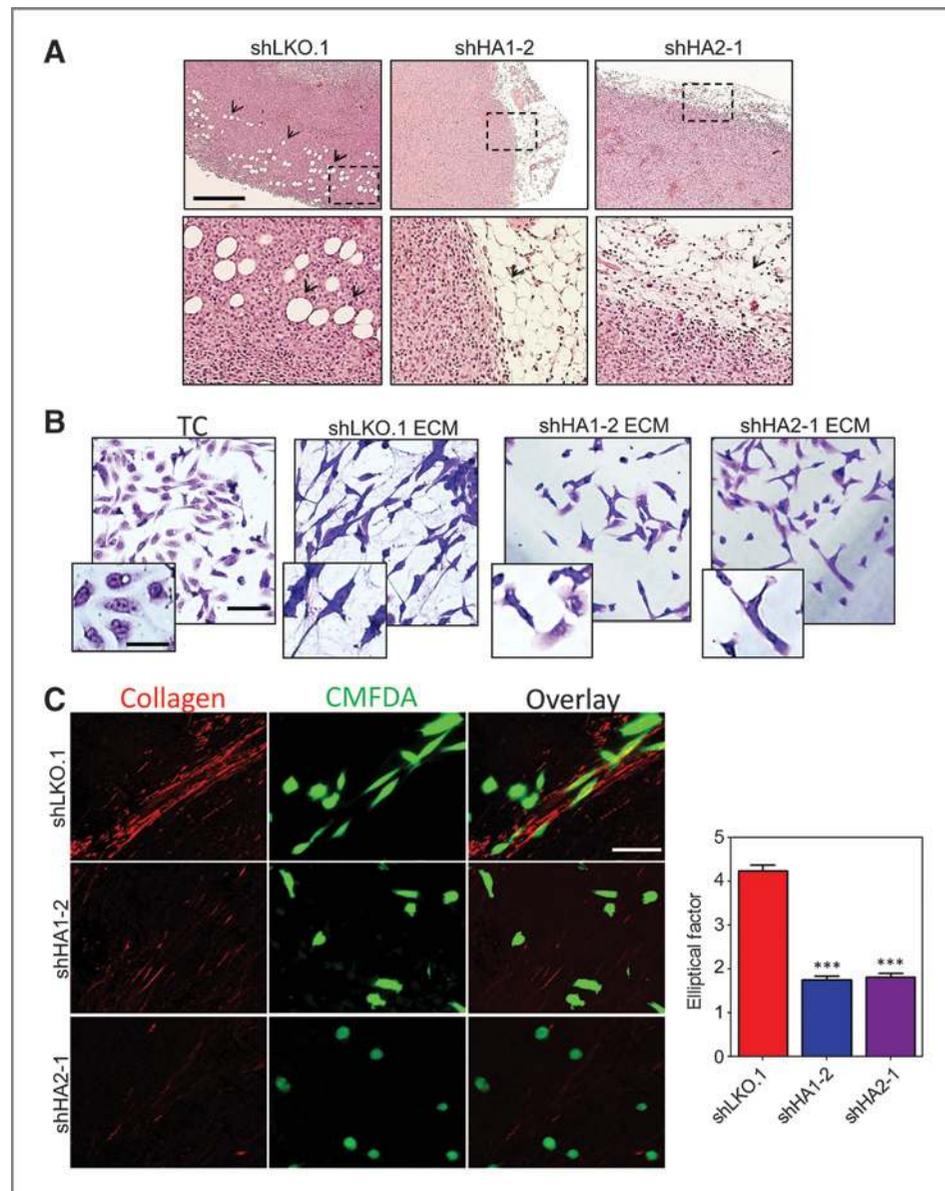
Collagen hydroxylases promote intravasation of breast cancer cells

We hypothesized that reduced cell invasion should result in reduced intravasation. To investigate the contribution of P4HA expression to intravasation, we determined the number of circulating tumor cells (CTC) following MFP injection. To construct a standard curve, we extracted total RNA from 0.5 mL of whole blood from nontumor-bearing mice mixed with 0, 100, or 400 MDA-MB-231 cells and measured human-specific rRNA levels (Fig. 5A). Human-specific rRNA levels in

whole blood of tumor-bearing mice were compared with the standard curve to quantify CTCs. P4HA knockdown significantly reduced the number of CTCs (Fig. 5B), suggesting that reduced invasion resulted in decreased intravasation of tumor cells.

To investigate the role of hydroxylase enzymes in premetastatic niche formation, we injected shEV-, shLOX-, shP4HA1-, or shP4HA2 subclones and assessed collagen crosslinking in the lung parenchyma [an early step in premetastatic niche formation that is mediated by LOX (9, 31, 44)]. Unlike LOX knockdown, P4HA1 or P4HA2 knockdown had no effect on premetastatic niche formation (Fig. 5C and Supplementary Fig. S5). This result is consistent with the role of collagen prolyl

Figure 4. Collagen prolyl hydroxylase expression promotes invasion. **A**, representative hematoxylin and eosin staining of the tumor/adipose tissue boundary. Scale bar, 200 μ m. **B**, crystal violet staining of MDA-MB-231 cells plated on tissue culture plastic (TC) or ECM-derived from indicated subclones. Scale bar, 100 μ m; inset scale bar, 25 μ m. **C**, CMFDA-labeled naïve MDA-MB-231 cells (green) were seeded on tumor sections and stained with picosirius red to image collagen fibers (red). Scale bar, 100 μ m. The elliptical factor (ratio of length to width) was calculated for 5-10 cells per tumor section and 5 sections per subclone. The mean \pm SEM values for the elliptical factor of cells seeded on sections of tumors derived from each of the three indicated subclones are shown in the bar graph; ***, $P < 0.001$ vs. shLKO.1 by ANOVA.



hydroxylases as intracellular, nonsecreted enzymes as compared with LOX, which is an extracellular enzyme that modifies collagen already present in the ECM.

Next, we assessed extravasation by directly injecting MDA-MB-231 subclones into the tail vein of mice. P4HA knockdown did not affect the ability of cells to extravasate (Fig. 5D and E). However, lung foci composed of P4HA knockdown cells were smaller (Fig. 5D) and contained less collagen (Fig. 5F) than control cell foci. Thus, P4HA knockdown significantly reduces intravasation but does not impair premetastatic niche formation or extravasation.

P4HA reexpression in HIF-deficient cells rescues collagen deposition and metastasis

To complement loss-of-function studies, MDA-MB-231-sh1/2 α cells were transduced with lentiviral vectors

encoding full-length P4HA1 (HA1) or P4HA2 (HA2), or with a control empty vector. MDA-MB-231-sh1/2 α +HA1 (or +HA2) cells expressed P4HA levels comparable with MDA-MB-231-EV cells exposed to 1% O₂ (Supplementary Fig. 6A). P4HA1 or P4HA2 overexpression in sh1/2 α cells increased tumor growth (Supplementary Fig. S6B and S6C), promoted metastasis (Supplementary Fig. S6D–S6F), recovered collagen content, and enhanced intravasation (Supplementary Fig. S6G–S6I). These data indicate that P4HA1 and P4HA2 are critical components of the HIF-dependent transcriptional response to hypoxia that promotes breast cancer metastasis. P4HA1 or P4HA2 overexpression could not fully compensate for the loss of HIF activity with respect to metastasis, which is consistent with the role of HIFs in the regulation of multiple genes involved in metastasis (8, 9).

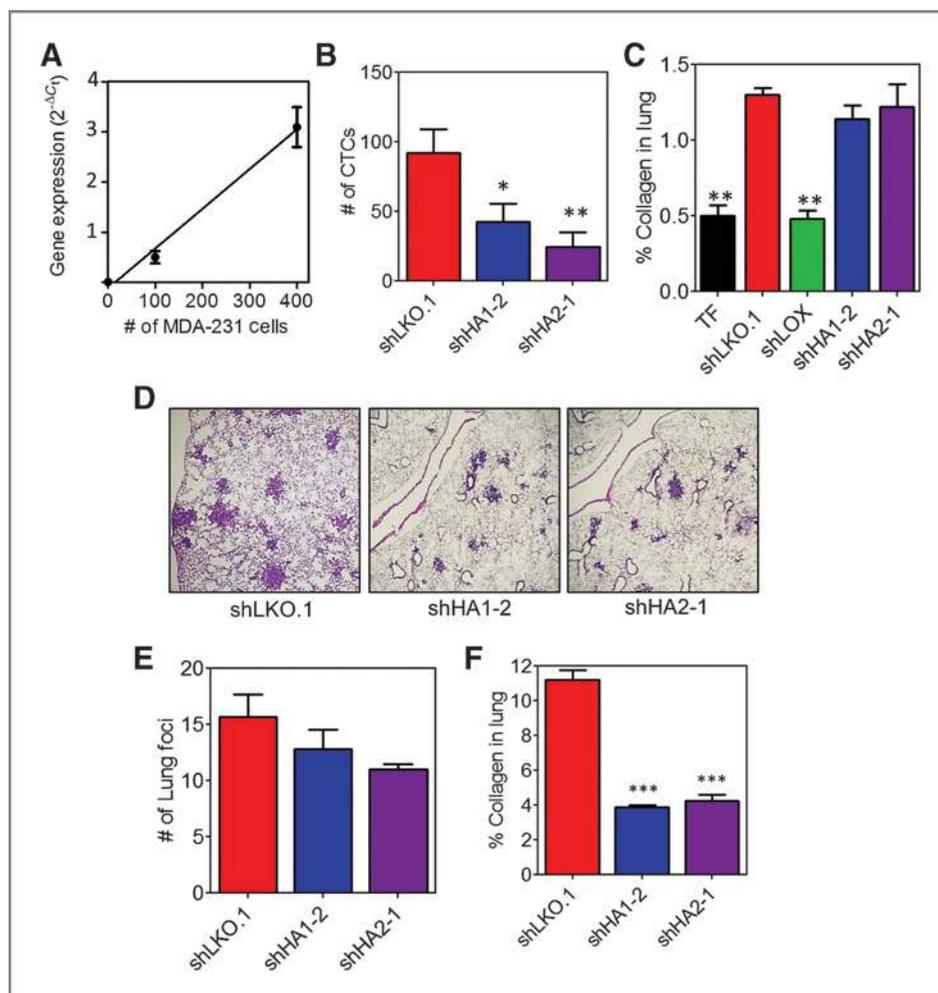


Figure 5. Collagen prolyl hydroxylase expression promotes intravasation. A, human rRNA expression from peripheral blood (0.5 mL) from nontumor-bearing NOD-SCID mice mixed with 0, 100, or 400 MDA-MB-231 cells. B, the number of tumor cells from the peripheral blood (0.5 mL) of tumor-bearing mice was determined using the standard curve in A (mean \pm SEM; $n = 8$, ANOVA). C, quantification of picosirius red staining of lung sections from tumor-free (TF) or tumor-bearing mice (mean \pm SEM; $n = 5$, ANOVA). D–F, mice received a tail vein injection of the indicated subclone and lung sections were subsequently analyzed 3 weeks after injection by staining with hematoxylin and eosin (D) for quantitation of metastatic foci per 4 \times field (E; mean \pm SEM; $n = 10$, ANOVA) and with picosirius red staining for quantitation of area occupied by collagen fibers (F; mean \pm SEM; $n = 5$, one-way ANOVA). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. shLKO.1.

P4HA expression correlates with human breast cancer prognosis

To investigate whether collagen prolyl hydroxylase expression has clinical significance for breast cancer prognosis, we analyzed *P4HA1* and *P4HA2* gene expression in human breast cancer using the Oncomine database (34), which revealed that P4HA levels were significantly greater in breast cancer tissue than in normal breast tissue (Fig. 6A). Results from an analysis of The Cancer Genome Atlas (<http://tcga-data.nci.nih.gov>) data were similar (Fig. 6B). *P4HA1* and *P4HA2* expression showed highly significant associations with breast cancer stage, was independent of hormone receptor status, but correlated with HER2 expression (Fig. 6C and Supplementary Table S3). *P4HA1* and *P4HA2* mRNA levels (35) were also significantly associated with decreased patient survival (Fig. 6D). The survival prediction was enhanced by further stratifying patients using both *P4HA1* and *P4HA2* expression. Thus, expression levels of collagen hydroxylases are prognostic in breast cancer, which is consistent with their essential role in promoting metastasis in the orthotopic transplantation model.

Therapeutic efficacy of collagen prolyl hydroxylase inhibition by DHB

Collagen prolyl hydroxylases are members of a superfamily of dioxygenases that use O_2 and α -ketoglutarate as substrates and are competitively inhibited by α -ketoglutarate analogs such as DHB (Fig. 7A; ref. 45). To evaluate the therapeutic efficacy of inhibiting collagen hydroxylases, we used DHB, which inhibits prolyl hydroxylase activity without affecting total protein synthesis or DNA replication (46). We found that DHB inhibited collagen secretion by cultured MDA-MB-231 cells in a dose-dependent manner (Fig. 7B). Treatment of MDA-MB-231 cells with DHB had only a modest inhibitory effect on cell proliferation *in vitro* (Supplementary Fig. S7A). Next, we established MFP tumors using MDA-MB-231-sh1/2 α +HA1 cells to mitigate any confounding effects of DHB on HIF-1 α levels (which are negatively regulated by the HIF prolyl hydroxylase PHD2) while maintaining levels of P4HA1. Systemic treatment of mice with 40 mg/kg per day of DHB led to modestly decreased primary tumor growth (Fig. 7C), but significantly reduced collagen content (Fig. 7D and E) and lung metastasis (Fig. 7F and G) without affecting body weight (Supplementary Fig. S7B). These results suggest that DHB (and

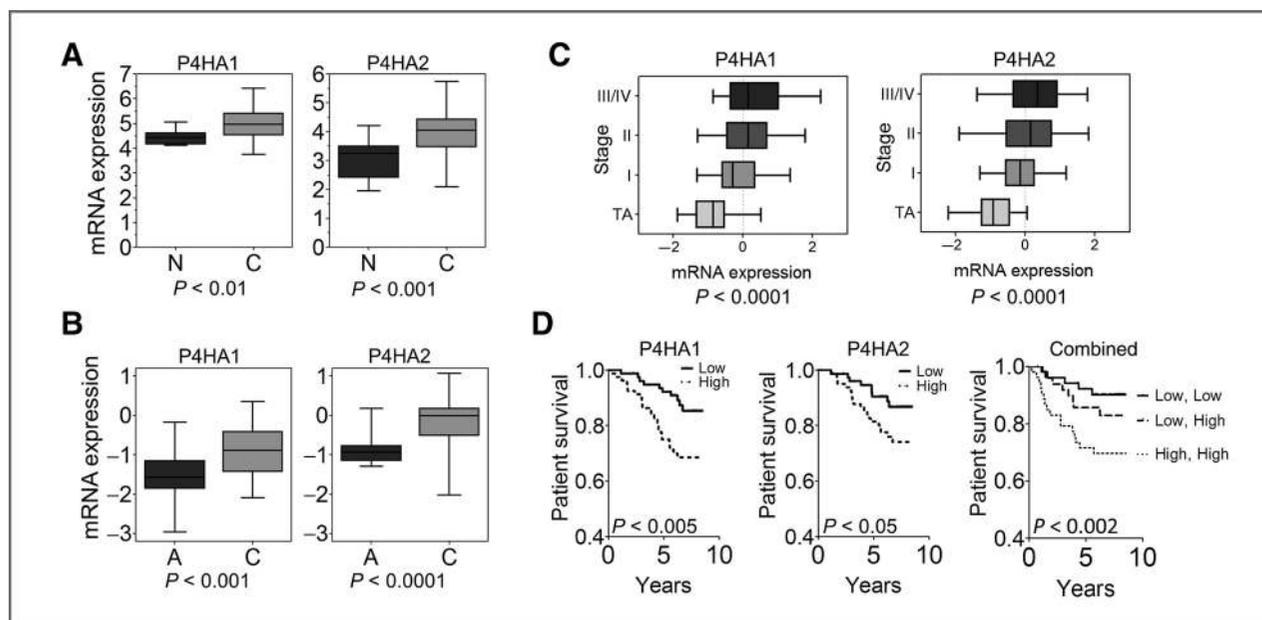


Figure 6. P4HA expression is associated with poor prognosis in breast cancer. A and B, P4HA1 and P4HA2 mRNA levels in normal breast (bar N; $n = 7$) and breast cancer (bar C; $n = 40$) tissues (A) or paired adjacent normal (bar A) versus breast cancer (bar C) tissues from The Cancer Genome Atlas ($n = 28$; B) are shown. Box displays 25th through 75th percentiles and whiskers represent the range of the data. C, P4HA1 and P4HA2 mRNA levels in breast cancer (stage = I, II, III/IV) normalized to the mean level in adjacent normal (stage = TA) from The Cancer Genome Atlas, are plotted as described above ($n = 458$; Pearson's correlation). D, Kaplan-Meier analysis of patient survival ($n = 159$) stratified by P4HA1 (left), P4HA2 (center), or both P4HA1 and P4HA2 (right) mRNA levels. High/low, gene expression above/below the median level.

possibly other prolyl hydroxylase inhibitors) may provide a novel pharmacologic strategy to inhibit metastasis, especially in patients with breast cancer with increased levels of P4HA mRNA demonstrable in tumor biopsies.

Discussion

Although several studies have reported increased collagen deposition during breast cancer progression (19, 41, 47), this is the first study to uncover a molecular mechanism linking intratumoral hypoxia, collagen deposition, and breast cancer invasion and metastasis. We showed that hypoxia-induced HIF-1 transcriptional activity upregulates collagen prolyl hydroxylase expression to dictate tumor collagen content, organization, density, and stiffness in breast cancer (Fig. 7H). We determined that collagen prolyl hydroxylases are essential for the metastasis of MDA-MB-231 and MDA-MB-435 breast cancer cells to the lungs of NOD/SCID mice by enhancing tissue invasion and intravasation. Moreover, we showed the prognostic significance of P4HA mRNA expression and identified a novel potential therapeutic intervention.

Collagen hydroxylases establish a tumor ECM that facilitates metastasis

Collagen content and organization have been shown to promote cancer progression by several mechanisms. Fibrillar collagen content affects the biophysical properties of the ECM (16–18). In our study, decreased P4HA expression reduced tumor stiffness and density by reducing the amount of collagen in the ECM. Increased breast cancer stiffness facilitates integrin clustering to promote focal adhesions, which drive invasion

(18). Collagen fiber alignment has been shown to play a critical role in directing the migration of tumor cells *in vivo* (25). We found that aligned collagen fibers were localized to perinecrotic (hypoxic) regions of tumors. Furthermore, we determined that cancer cells aligned themselves along these fibers, adopting a spindle-shaped morphology. Cancer cells seeded on P4HA knockdown tumor sections, in which collagen deposition was significantly decreased, remained rounded and lacked directionality. Although the importance of collagen content in the tumor microenvironment has been well reported (48), this study is the first to highlight a pathophysiologic mechanism (i.e., intratumoral hypoxia) that leads to increased collagen deposition during breast cancer progression. Whereas prior studies have focused on the role of stromal cells, the present study shows that breast cancer cells may also make a critical contribution to ECM remodeling during cancer progression.

Collagen hydroxylases promote intravasation but not extravasation or premetastatic niche formation

P4HA1 or P4HA2 knockdown prevented cancer cells from invading adjacent fat tissue, decreased the number of CTCs, and blocked lung metastasis, showing the role of these collagen modifying enzymes in promoting hematogenous dissemination of breast cancer cells to the lungs and possibly other organs. In contrast, P4HA knockdown had no effect on the number of lung foci that formed after direct injection into the tail vein of the mice. Taken together, these data indicate that P4HA1 and P4HA2 play an important role in promoting intravasation by enhancing tissue invasion, whereas they do not affect extravasation. Unlike the secreted enzymes, LOX, LOXL2, and LOXL4, which have been shown to influence

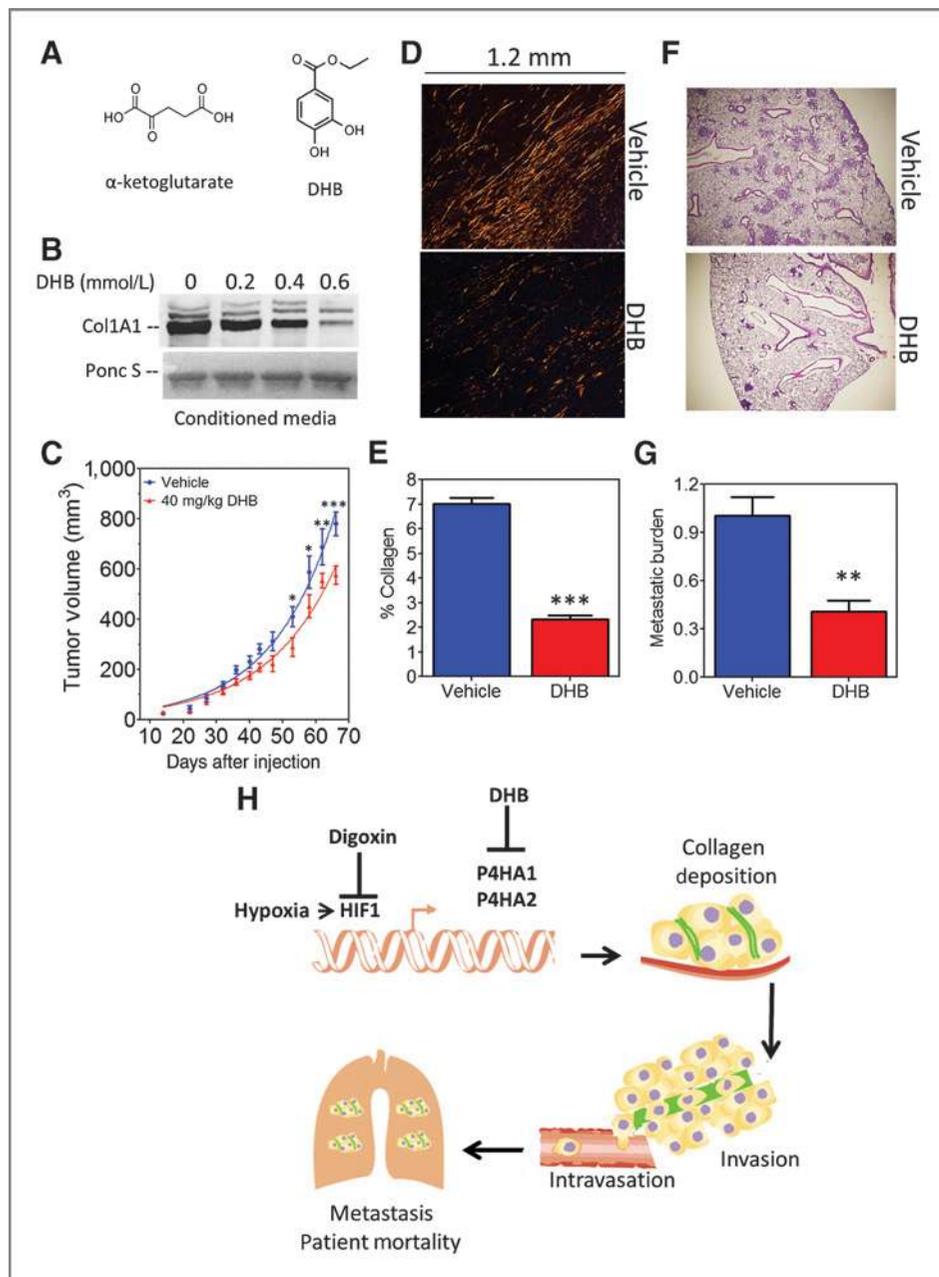


Figure 7. Inhibiting collagen prolyl hydroxylase activity using DHB inhibits tumor growth, collagen deposition, and metastasis.

A, chemical structures of α -ketoglutarate and ethyl 3,4 dihydroxybenzoate (DHB). **B**, immunoblot assay for collagen 1A1 in conditioned media of MDA-MB-231 cells treated with DHB for 24 hours. Ponceau S, Ponceau S staining of membrane. **C**, NOD-SCID mice bearing MDA-MB-231 orthografts received daily i.p. injections of DHB or vehicle and tumor volume (mean \pm SEM; $n = 5$; ANOVA) was determined. **D** and **E**, tumor sections were stained with picosirius red and collagen was quantified by image analysis under polarized light (mean \pm SEM; $n = 5$; Student t test). **F**, lung sections (4×3 mm) were stained with hematoxylin and eosin. **G**, human genomic DNA content in mouse lungs was determined (mean \pm SEM; $n = 5$; Student t test). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. vehicle treatment. **H**, hypoxia-induced collagen prolyl hydroxylase expression promotes collagen deposition and breast cancer metastasis.

metastatic sites by remodeling collagen before the arrival of cancer cells (9, 31), collagen hydroxylases did not affect pre-metastatic niche formation in the lung.

HIF-1 regulates multiple steps in collagen biogenesis

Prior studies focused on the late stages of collagen maturation and crosslinking mediated by the LOX family of extracellular enzymes (9, 31, 44, 49). The present study highlights the importance of the initial intracellular steps in collagen biogenesis, which control the amount of collagen available for crosslinking. Taken together, HIF-1 affects both early (intracellular) and late (extracellular) steps in collagen fibril forma-

tion. This is important because fibrotic breast cancers have the poorest prognosis and highest rate of recurrence (50) and in mouse models, increased type I collagen is associated with increased tumor growth and lung metastasis (19).

In addition to prolyl hydroxylation, the other major post-translational modification of procollagen prior to secretion is lysyl hydroxylation, which is also induced by hypoxia in a HIF-1-dependent manner and is required for breast cancer metastasis (51). Knockdown of prolyl hydroxylase expression in breast cancer cells blocks collagen deposition in the primary tumor, whereas knockdown of lysyl hydroxylase expression has no effect on collagen deposition but specifically impairs

collagen fiber formation (51). Thus, the coordinate transcriptional activation of genes encoding prolyl and lysyl hydroxylases in hypoxic breast cancer cells is critical for ECM remodeling that promotes local invasion as well as lymphatic and hematogenous metastasis.

These findings, taken together with the dramatic effect of P4HA1 or P4HA2 knockdown, indicate that collagen prolyl hydroxylases represent an important new therapeutic target for the prevention of metastasis in breast cancer. The data also support a novel paradigm of cancer cells directly contributing to the prometastatic microenvironment of the primary tumor by promoting ECM production. Given that cancer-associated fibroblasts and myofibroblasts contribute to ECM assembly, drugs targeting collagen hydroxylase expression, ECM maintenance, and/or HIF activity must effectively target cancer as well as stromal cells. Drugs such as digoxin that inhibit HIF-1 activity may be beneficial because they inhibit tumor fibrosis by blocking multiple steps in collagen biogenesis as well as affecting other critical aspects of the metastatic process (8, 9, 44, 52).

Inhibitors of collagen prolyl hydroxylases or HIF-1 as potential therapeutics for cancer

The MDA-MB-231 and MDA-MB-435 cell lines were derived from triple-negative breast cancers, which lack significant expression of the estrogen receptor, progesterone receptor, or HER2. A recent analysis of more than 500 samples of patients with breast cancer conducted by the Cancer Genome Atlas Network found a significant association between HIF-1 pathway activation and triple-negative breast cancers (53) highlighting the potential impact of

inhibitors of collagen prolyl hydroxylases or HIF-1 on this patient subset, which responds poorly to currently available therapies (54).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: D.M. Gilkes, G.L. Semenza

Development of methodology: D.M. Gilkes, P. Chaturvedi, S. Bajpai, H. Wei, S. Pitcairn, H. Wei, D. Wirtz, G.L. Semenza

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.M. Gilkes, S. Bajpai, M.E. Hubbi, D. Wirtz, G.L. Semenza

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.M. Gilkes, S. Bajpai, D. Wirtz, G.L. Semenza

Writing, review, and/or revision of the manuscript: D.M. Gilkes, M.E. Hubbi, G.L. Semenza

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.C. Wong

Study supervision: D. Wirtz, G.L. Semenza

Acknowledgments

The authors thank Jasper Chen for assistance with animal experiments, Huaifeng Zhang for training and advice, and Karen Padgett of Novus Biologicals for generous gifts of antibodies against HIF-2 α , P4HA1, P4HA2, and COL1A1.

Grant Support

This work was supported in part by the Johns Hopkins Physical Sciences-Oncology Center through grant U54-CA143868 from the National Cancer Institute. D.M. Gilkes is the recipient of a Susan G. Komen Breast Cancer Foundation Post-Doctoral Fellowship Award.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received October 23, 2012; revised January 10, 2013; accepted February 23, 2013; published OnlineFirst March 28, 2013.

References

- Brahimi-Horn MC, Chiche J, Pouyssegur J. Hypoxia and cancer. *J Mol Med* 2007;85:1301-7.
- Dewhirst MW. Intermittent hypoxia furthers the rationale for hypoxia-inducible factor-1 targeting. *Cancer Res* 2007;67:854-5.
- Semenza GL. Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. *Oncogene* 2010;29:625-34.
- Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O₂ tension. *Proc Natl Acad Sci U S A* 1995;92:5510-4.
- Hiraga T, Kizaka-Kondoh S, Hirota K, Hiraoka M, Yoneda T. Hypoxia and hypoxia-inducible factor-1 expression enhance osteolytic bone metastases of breast cancer. *Cancer Res* 2007;67:4157-63.
- Li L, Lin X, Staver M, Shoemaker A, Semizarov D, Fesik SW, et al. Evaluating hypoxia-inducible factor-1 α as a cancer therapeutic target via inducible RNA interference *in vivo*. *Cancer Res* 2005;65:7249-58.
- Liao D, Corle C, Seagroves TN, Johnson RS. Hypoxia-inducible factor-1 α is a key regulator of metastasis in a transgenic model of cancer initiation and progression. *Cancer Res* 2007;67:563-72.
- Zhang H, Wong CC, Wei H, Gilkes DM, Korangath P, Chaturvedi P, et al. HIF-1-dependent expression of angiopoietin-like 4 and L1CAM mediates vascular metastasis of hypoxic breast cancer cells to the lungs. *Oncogene* 2012;31:1757-70.
- Wong CC, Gilkes DM, Zhang H, Chen J, Wei H, Chaturvedi P, et al. Hypoxia-inducible factor 1 is a master regulator of breast cancer metastatic niche formation. *Proc Natl Acad Sci U S A* 2011;108:16369-74.
- Bos R, van der Groep P, Greijer AE, Shvarts A, Meijer S, Pinedo HM, et al. Levels of hypoxia-inducible factor-1 α independently predict prognosis in patients with lymph node negative breast carcinoma. *Cancer* 2003;97:1573-81.
- Schindl M, Schoppmann SF, Samonigg H, Hausmaninger H, Kwasny W, Gnant M, et al. Overexpression of hypoxia-inducible factor 1 α is associated with an unfavorable prognosis in lymph node-positive breast cancer. *Clin Cancer Res* 2002;8:1831-7.
- Generali D, Berruti A, Brizzi MP, Campo L, Bonardi S, Wigfield S, et al. Hypoxia-inducible factor-1 α expression predicts a poor response to primary chemoendocrine therapy and disease-free survival in primary human breast cancer. *Clin Cancer Res* 2006;12:4562-8.
- Yamamoto Y, Ibusuki M, Okumura Y, Kawasoe T, Kai K, Iyama K, et al. Hypoxia-inducible factor 1 α is closely linked to an aggressive phenotype in breast cancer. *Breast Cancer Res Treat* 2008;110:465-75.
- Dales JP, Garcia S, Meunier-Carpentier S, Andrac-Meyer L, Haddad O, Lavaut MN, et al. Overexpression of hypoxia-inducible factor HIF-1 α predicts early relapse in breast cancer: retrospective study in a series of 745 patients. *Int J Cancer* 2005;116:734-9.
- Helczynska K, Larsson AM, Holmquist Mengelbier L, Bridges E, Fredlund E, Borgquist S, et al. Hypoxia-inducible factor-2 α correlates to distant recurrence and poor outcome in invasive breast cancer. *Cancer Res* 2008;68:9212-20.
- Paszek MJ, Zahir N, Johnson KR, Lakins JN, Rozenberg GI, Gefen A, et al. Tensional homeostasis and the malignant phenotype. *Cancer Cell* 2005;8:241-54.
- Lopez JL, Mouw JK, Weaver VM. Biomechanical regulation of cell orientation and fate. *Oncogene* 2008;27:6981-93.

18. Levental KR, Yu H, Kass L, Lakins JN, Egeblad M, Erler JT, et al. Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell* 2009;139:891–906.
19. Provenzano PP, Inman DR, Eliceiri KW, Knittel JG, Yan L, Rueden CT, et al. Collagen density promotes mammary tumor initiation and progression. *BMC Med* 2008;6:11.
20. Lopez JI, Kang I, You WK, McDonald DM, Weaver VM. *In situ* force mapping of mammary gland transformation. *Integr Biol (Camb)* 2011;3:910–21.
21. Junqueira LC, Bignolas G, Brentani RR. Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. *Histochem J* 1979;11:447–55.
22. Maglione JE, McGoldrick ET, Young LJ, Namba R, Gregg JP, Liu L, et al. Polyomavirus middle T-induced mammary intraepithelial neoplasia outgrowths: single origin, divergent evolution, and multiple outcomes. *Mol Cancer Ther* 2004;3:941–53.
23. Lin EY, Jones JG, Li P, Zhu L, Whitney KD, Muller WJ, et al. Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases. *Am J Pathol* 2003;163:2113–26.
24. Provenzano PP, Eliceiri KW, Campbell JM, Inman DR, White JG, Keely PJ. Collagen reorganization at the tumor-stromal interface facilitates local invasion. *BMC Med* 2006;4:38.
25. Wyckoff JB, Wang Y, Lin EY, Li JF, Goswami S, Stanley ER, et al. Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. *Cancer Res* 2007;67:2649–56.
26. Wang W, Wyckoff JB, Frohlich VC, Oleynikov Y, Huttelmaier S, Zavadil J, et al. Single cell behavior in metastatic primary mammary tumors correlated with gene expression patterns revealed by molecular profiling. *Cancer Res* 2002;62:6278–88.
27. Annunen P, Helaakoski T, Myllyharju J, Veijola J, Pihlajaniemi T, Kivirikko KI. Cloning of the human prolyl 4-hydroxylase α subunit isoform α (II) and characterization of the type II enzyme tetramer. The α (I) and α (II) subunits do not form a mixed α (I) α (II) β 2 tetramer. *J Biol Chem* 1997;272:17342–8.
28. Kukkola L, Hieta R, Kivirikko KI, Myllyharju J. Identification and characterization of a third human, rat, and mouse collagen prolyl 4-hydroxylase isoenzyme. *J Biol Chem* 2003;278:47685–93.
29. Myllyharju J. Prolyl 4-hydroxylases, the key enzymes of collagen biosynthesis. *Matrix Biol* 2003;22:15–24.
30. Myllyharju J, Kivirikko KI. Collagens, modifying enzymes and their mutations in humans, flies and worms. *Trends Genet* 2004;20:33–43.
31. Erler JT, Bennewith KL, Cox TR, Lang G, Bird D, Koong A, et al. Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche. *Cancer Cell* 2009;15:35–44.
32. Berg RA. Determination of 3- and 4-hydroxyproline. *Methods Enzymol* 1982;82:372–98.
33. Krishnamachary B, Semenza GL. Analysis of hypoxia-inducible factor 1 α expression and its effects on invasion and metastasis. *Methods Enzymol* 2007;435:347–54.
34. Richardson AL, Wang ZC, De Nicolo A, Lu X, Brown M, Miron A, et al. X chromosomal abnormalities in basal-like human breast cancer. *Cancer Cell* 2006;9:121–32.
35. Pawitan Y, Bjohle J, Amler L, Borg AL, Egyhazi S, Hall P, et al. Gene expression profiling spares early breast cancer patients from adjuvant therapy: derived and validated in two population-based cohorts. *Breast Cancer Res* 2005;7:R953–64.
36. Polyak K, Kalluri R. The role of the microenvironment in mammary gland development and cancer. *Cold Spring Harb Perspect Biol* 2010;2:a003244.
37. Nyberg P, Salo T, Kalluri R. Tumor microenvironment and angiogenesis. *Front Biosci* 2008;13:6537–53.
38. Tse JC, Kalluri R. Mechanisms of metastasis: epithelial-to-mesenchymal transition and contribution of tumor microenvironment. *J Cell Biochem* 2007;101:816–29.
39. Zhang H, Qian DZ, Tan YS, Lee K, Gao P, Ren YR, et al. Digoxin and other cardiac glycosides inhibit HIF-1 α synthesis and block tumor growth. *Proc Natl Acad Sci U S A* 2008;105:19579–86.
40. Gorres KL, Edupuganti R, Krow GR, Raines RT. Conformational preferences of substrates for human prolyl 4-hydroxylase. *Biochemistry* 2008;47:9447–55.
41. Conklin MW, Eickhoff JC, Riching KM, Pehlke CA, Eliceiri KW, Provenzano PP, et al. Aligned collagen is a prognostic signature for survival in human breast carcinoma. *Am J Pathol* 2011;178:1221–32.
42. Cassereau L, DuFort CC, Weaver VM. Morphogenesis: laying down the tracks. *Nat Mater* 2012;11:490–2.
43. Friedl P, Wolf K. Tumor cell invasion and migration: diversity and escape mechanisms. *Nat Rev Cancer* 2003;3:362–74.
44. Wong CC, Zhang H, Gilkes DM, Chen J, Wei H, Chaturvedi P, et al. Inhibitors of hypoxia-inducible factor 1 block breast cancer metastatic niche formation and lung metastasis. *J Mol Med (Berl)* 2012;90:803–15.
45. Rose NR, McDonough MA, King ON, Kawamura A, Schofield CJ. Inhibition of 2-oxoglutarate dependent oxygenases. *Chem Soc Rev* 2011;40:4364–97.
46. Nandan D, Clarke EP, Ball EH, Sanwal BD. Ethyl-3,4-dihydroxybenzoate inhibits myoblast differentiation: evidence for an essential role of collagen. *J Cell Biol* 1990;110:1673–9.
47. Provenzano PP, Inman DR, Eliceiri KW, Keely PJ. Matrix density-induced mechanoregulation of breast cell phenotype, signaling and gene expression through a FAK-ERK linkage. *Oncogene* 2009;28:4326–43.
48. Hynes RO. The extracellular matrix: not just pretty fibrils. *Science* 2009;326:1216–9.
49. Erler JT, Bennewith KL, Nicolau M, Dornhofer N, Kong C, Le QT, et al. Lysyl oxidase is essential for hypoxia-induced metastasis. *Nature* 2006;440:1222–6.
50. Hasebe T, Tsuda H, Tsubono Y, Imoto S, Mukai K. Fibrotic focus in invasive ductal carcinoma of the breast: a histopathological prognostic parameter for tumor recurrence and tumor death within three years after the initial operation. *Jpn J Cancer Res* 1997;88:590–9.
51. Gilkes DM, Bajpai S, Wong CC, Chaturvedi P, Hubbi ME, Wirtz D, Semenza GL. Procollagen lysyl hydroxylase 2 is essential for hypoxia-induced breast cancer metastasis. *Mol Cancer Res* 2013;11:456–66.
52. Schito L, Rey S, Tafani M, Zhang H, Wong CC-L, Russo A, et al. Hypoxia-inducible factor 1-dependent expression of platelet-derived growth factor B promotes lymphatic metastasis of hypoxic breast cancer cells. *Proc Natl Acad Sci U S A* 2012;109:E2707–16.
53. Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumors. *Nature* 2012;490:61–70.
54. Pal SK, Childs BH, Pegram M. Triple negative breast cancer metastasis: markers and models. *Nat Rev Cancer* 2011;12:627–36.

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

Collagen Prolyl Hydroxylases Are Essential for Breast Cancer Metastasis

Daniele M. Gilkes, Pallavi Chaturvedi, Saumendra Bajpai, et al.

Cancer Res 2013;73:3285-3296. Published OnlineFirst March 28, 2013.

Updated version Access the most recent version of this article at:
doi:[10.1158/0008-5472.CAN-12-3963](https://doi.org/10.1158/0008-5472.CAN-12-3963)

Supplementary Material Access the most recent supplemental material at:
<http://cancerres.aacrjournals.org/content/suppl/2013/03/28/0008-5472.CAN-12-3963.DC1>

Cited articles This article cites 54 articles, 20 of which you can access for free at:
<http://cancerres.aacrjournals.org/content/73/11/3285.full#ref-list-1>

Citing articles This article has been cited by 41 HighWire-hosted articles. Access the articles at:
<http://cancerres.aacrjournals.org/content/73/11/3285.full#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
<http://cancerres.aacrjournals.org/content/73/11/3285>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.