Hypoxia-Dependent Modification of Collagen Networks Promotes Sarcoma Metastasis

ABSTRACT

Intratumoral hypoxia and expression of hypoxia-inducible factor-1α (HIF-1α) correlate with metastasis and poor survival in patients with sarcoma. We show here that hypoxia controls sarcoma metastasis through a novel mechanism wherein HIF-1α enhances expression of the intracellular enzyme procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2). We show that loss of HIF-1α or PLOD2 expression disrupts collagen modification, cell migration, and pulmonary metastasis (but not primary tumor growth) in allograft and autochthonous LSL-KrasG12D/+; Trp53fl/fl murine sarcoma models. Furthermore, ectopic PLOD2 expression restores migration and metastatic potential in HIF-1α-deficient tumors, and analysis of human sarcomas reveals elevated HIF1A and PLOD2 expression in metastatic primary lesions. Pharmacologic inhibition of PLOD enzymatic activity suppresses metastases. Collectively, these data indicate that HIF-1α controls sarcoma metastasis through PLOD2-dependent collagen modification and organization in primary tumors. We conclude that PLOD2 is a novel therapeutic target in sarcomas and successful inhibition of this enzyme may reduce tumor cell dissemination.

SIGNIFICANCE: Undifferentiated pleomorphic sarcoma (UPS) is a commonly diagnosed and particularly aggressive sarcoma subtype in adults, which frequently and fatally metastasizes to the lung. Here, we show the potential use of a novel therapeutic target for the treatment of metastatic UPS, specifically the collagen-modifying enzyme PLOD2. Cancer Discov; 3(10); 1190–1205. © 2013 AACR.

See related commentary by Vanharanta and Massagué, p. 1103.

INTRODUCTION

Sarcomas are diagnosed in 200,000 people worldwide annually; approximately 40% of these patients ultimately succumb to lethal metastases (1, 2). Current treatment options available to patients with sarcoma are standard surgical resection, radiotherapy, and chemotherapy, and limited molecular analyses of human sarcomas have proven an impediment to developing novel, sarcoma-specific, therapeutic options. Although genetic lesions affecting multiple signaling pathways (Kras, Pten, Ptp1b, and p53) have been identified in distinct soft tissue sarcoma subtypes (3, 4), relatively little is understood about the downstream molecular mechanisms that drive sarcomagenesis and progression. Furthermore, soft-tissue sarcomas encompass more than 50 distinct disease subtypes (e.g., fibrosarcoma, liposarcoma, rhabdomyosarcoma, etc.), all of which undergo constant reevaluation as developing technologies allow for more thorough characterization of each malignancy (5). As in other tumors, aggressive metastatic behavior in sarcomas is frequently associated with high levels of tumor cell dedifferentiation (6). Consistent with this observation, undifferentiated pleomorphic sarcoma (UPS) has been identified as one of the most frequently diagnosed subtypes, which commonly results in lethal pulmonary metastases. Current data suggest that UPS may not represent a distinct sarcoma subtype, but rather a collection of phenotypes common to other sarcomas in their more advanced stages (7). It has been argued that UPS exists along a continuum, wherein unique sarcoma subtypes become increasingly undifferentiated as they worsen in stage and grade until their tissue/cell type of origin is no longer discernible (7). Regardless of whether UPS is ultimately shown to be distinct or a culmination of sarcoma progression, these tumors are associated with poor clinical outcome due to metastases. As metastasis, particularly to the lungs, remains the most common cause of sarcoma-associated death, elucidating the molecular and cellular mechanisms controlling the sarcoma cell dissemination is critical to the development of effective therapeutic strategies to treat these cancers.

The development of successful therapeutic interventions for sarcoma will depend on the ability to accurately model UPS and other subtypes. One mouse model for investigating UPS uses simultaneous Cre-dependent expression of oncogenic KrasG12D and deletion of Trp53 in the left gastrocnemius muscle (8). These genetic changes occur frequently in sarcoma, and the murine tumors that develop recapitulate human UPS morphologically, histologically, and genetically (8, 9). Most importantly, primary tumors that develop in this autochthonous model successfully metastasize to the lung, mirroring human UPS. Furthermore, subcutaneous allografts of murine UPS cells will also metastasize to the lung within several weeks of implantation. Combined, these approaches allow for the investigation of molecular mechanisms that govern primary UPS formation and pulmonary metastases.
The available clinical data indicate that high levels of intratumoral hypoxia and hypoxia-inducible factor-1α (HIF-1α) expression are among the most important predictors of metastatic potential in patients with sarcoma, although the underlying mechanisms for this correlation are unknown (10, 11). Metastasis is a complex multistep process wherein tumor cells are driven, in part by lack of oxygen and nutrients, to abandon their tissue of origin and colonize distant sites (12). For example, hypoxia has been shown to promote release of tumor cell–derived lysyl oxidase (LOX), an HIF-1α target that remodels collagen in the extracellular matrix (ECM) of remote sites, thereby contributing to the establishment of the “pre-metastatic niche” (13) in murine breast cancer models. Whether similar, or distinct, the cellular mechanisms that regulate sarcoma metastasis are as yet unknown.

Collagen is the most abundant structural component of the ECM and is aberrantly regulated in cancer at the levels of expression, posttranslational modification, deposition, and degradation (14). Consistent with their mesenchymal origins, primary sarcomas produce and secrete large amounts of collagen, generating extensive extracellular collagen “highways.” These networks act as support scaffolds, facilitating tumor cell migration toward blood vessels and promoting their ability to escape the primary lesion (15–21). Mature collagen is formed by a series of enzymatic posttranslational modifications of immature collagen polypeptides (22–24), although the factors required to establish and maintain collagen networks in sarcomas are not clear. Recently, HIF-1α has been shown to regulate expression of prolyl hydroxylases and the endoplasmic reticulum–associated enzyme procollagen-lysine, 2-oxoglutarate 5-dioxygenase (PLOD2), also referred to as lysyl hydroxylase 2 (LH2; refs. 25, 26). The primary function of PLOD2 is the initiation of lysine hydroxylation of collagen molecules (27–29). Hydroxylysines form carbohydrate attachment sites and are essential for the stability of collagen cross-links (reviewed in ref. 22). Cross-linked collagen assemblies into a triple helix, departs the endoplasmic reticulum, and is then cleaved for assembly into fibrils. Prolyl and lysyl hydroxylations are crucial for the formation of normal mature collagen. Mutations in PLOD2 cause the autosomal recessive disorder Bruck syndrome, in which patients suffer osteoporosis, scoliosis, and joint contractures due to underhydroxylated collagen I (29); however, very little is known about the role of PLOD2 in tumorigenesis. Furthermore, the majority of research investigating the contribution of collagen and collagen-modifying enzymes to metastasis has been conducted on epithelial cell–derived tumors, primarily breast cancer (13, 30). These processes remain understudied in mesenchymal tumors, including sarcomas.

Here, we investigate the role of HIF-1α and PLOD2 in sarcoma using samples from human patients and genetically engineered mouse models that faithfully recapitulate key aspects of human UPS. We show that HIF-1α–dependent upregulation of PLOD2, but not LOX, is observed in metastatic human sarcomas and is essential for the creation of collagen networks in primary murine tumors and subsequent metastasis to the lung. Importantly, minoxidil-mediated PLOD inhibition decreased pulmonary metastasis in our murine allograft sarcoma model, suggesting that PLOD inhibition may prove a useful therapeutic intervention. Our findings indicate that intratumoral hypoxia and HIF-1α–dependent PLOD2 transcription promote sarcoma metastasis by modifying the collagen component of the ECM in primary tumors and stimulating sarcoma cell migration. Furthermore, these data indicate that HIF-1α confers distinct, tumor type–dependent effects on metastasis. Specifically, although HIF-1α–driven LOX and PLOD2 expression have been shown to modify the premetastatic niche in breast cancers (13, 31), PLOD2, but not LOX, modifies the collagen network in primary sarcomas, with consequent effects on tumor cell migration and metastasis. Finally, we have shown that PLOD2 is a credible and druggable therapeutic target in premetastatic sarcoma.

RESULTS
Elevated HIF-1α and PLOD2 Correlate with Sarcoma Metastasis, but Not Primary Tumor Formation, in Human and Autochthonous Murine Tumors

To determine whether HIF-1α–dependent upregulation of PLOD2 could promote metastasis in primary human sarcomas, we compared relative gene expression based on microarray analysis of human metastatic and nonmetastatic UPS and fibrosarcomas obtained before therapeutic intervention (32). HIF1A and PLOD2 expression was selectively elevated in metastatic tumors (Fig. 1A; left and middle); in contrast, expression of PLOD1, a closely related isoform of PLOD2, and LOX, another HIF-1α transcriptional target and collagen-modifying enzyme, was not significantly altered (data not shown). Furthermore, quantitative real-time PCR (qRT-PCR) analysis of RNA obtained from an independent cohort of human UPS tumors showed that PLOD2 levels are significantly higher in metastatic tumors relative to those that failed to metastasize (Fig. 1A, right). These data suggest that HIF-1α–mediated PLOD2 expression is associated with sarcoma metastasis.

We used the genetically engineered murine Lsl-KrasG12D/+; Trp53fl/fl (KP) model of UPS (8, 9) to investigate the effects of HIF–1α and its target genes on soft-tissue sarcoma development. In this model, injection of adeno-virus-expressing Cre recombinase (Adenex-Cre) into the left gastrocnemius muscle results in KrasG12D expression and Trp5 deletion, producing sarcomas within approximately 8 weeks. We also crossed KP mice to Hif1a−/− animals to generate the Lsl-KrasG12D/+; Trp53−/−; Hif1a−/− “KPH” strain, in which HIF–1α is deleted in the KrasG12D-expressing, p53-deficient tumors. Genetic analysis showed highly effective Cre-dependent recombination of Hif1a−/− alleles in the resulting sarcomas (Fig. 1B). KP and KPH animals developed tumors of similar size and latency, indicating that loss of HIF–1α did not alter primary tumor formation (Fig. 1C) or growth (Fig. 1D). However, HIF–1α deletion dramatically reduced the occurrence of pulmonary metastasis in this model, indicating that HIF–1α specifically modulates tumor cell dissemination in sarcomas (Fig. 1E). Analysis of primary sarcomas by Masson’s Trichrome staining of KP and KPH tumors revealed that HIF–1α deletion significantly alters deposited collagen (Fig. 1F). No collagen fibers were found intersecting blood vessels in KPH tumors, whereas in KP tumors, long strands of collagen with associated tumor cells were observed invading the vasculature (Fig. 1F, arrow). Of note, HIF–1α loss had no significant effect on sarcoma vessel density or perfusion (Supplementary Fig. S1A), indicating...
HIF-1α and PLOD2 Are Key Modulators of Sarcoma Metastasis

Figure 1. HIF-1α is an important regulator of metastasis in an autochthonous, genetic model of UPS, potentially via PLOD2 modulation. A, left and middle, relative gene expression in human metastatic (N = 5) and nonmetastatic (N = 8) UPS and fibrosarcoma in patients treated at Massachusetts General Hospital (Boston, MA; ref. 32). HIF1A (P = 0.0312) and PLOD2 (P = 0.0011) were significantly upregulated in metastatic sarcomas. Right, qRT-PCR analysis of 10 human UPS patient samples treated at the University of Pennsylvania (Philadelphia, PA); P = 0.044. B, mouse models of sarcoma. KP and KPH genotyping showed effective recombination of Hif1a alleles in Adeno-Cre–initiated tumors. C, mice remained tumor free for roughly 40 days; by 90 days, all of the mice had developed palpable tumors (volume = 200 mm³): KP (n = 30), KPH (n = 20), P = 0.3755. D, primary tumor size. Two weeks after tumors were palpable, they had grown twofold to eightfold larger, but there was no difference between KP (n = 7) and KPH (n = 9) tumor growth; P = 0.7342. E, metastasis-free survival in KP (n = 33) and KPH (n = 28) mice; P = 0.0456. Lung metastases were confirmed histologically. F, Masson’s Trichrome and picrosirius red staining of tumor nest areas and blood vessels in primary KP and KPH tumors. Deletion of HIF-1α alters collagen in KPH tumors. Masson’s Trichrome stains collagen fibers blue. Cells were counterstained in red with Weigert’s hematoxylin; scale bar, 50 μm. G, Western blot analyses of sarcoma cells derived from KP and KPH tumors. Expression of HIF-1α and PLOD2 proteins is hypoxia-inducible and abolished when HIF-1α is deleted. H, qRT-PCR analysis of two individually derived KP and KPH cell lines. Plo2d mRNA transcription is induced under hypoxic conditions in control cells (KP1, P = 0.0284; and KP2, P = 0.0391). Deletion of HIF-1α abolished hypoxia-induced Plo2d mRNA levels. I, Western blot analysis of PLOD2 expression in KIA cells, and J, HT-1080 cells. K, qRT-PCR analysis of two individually derived KIA cells. Expression of HIF-1α and Plo2d is hypoxia-inducible (Plo2d qRT-PCR, P = 0.0284) and is abolished when HIF-1α is deleted (Plo2d qRT-PCR, P = 0.0006). L, HT-1080 cells were evaluated by qRT-PCR as in K. I. Expression of Hif1a and Plo2d is hypoxia-inducible (Plo2d qRT-PCR, P = 0.0006) and is abolished when HIF-1α is deleted (Plo2d qRT-PCR, P = 0.0210). WT, wild-type; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
that the vasculature is unaffected. These data suggest that the vasculature in KPH tumors is functioning similarly to control KP tumors and is unlikely to be responsible for the decrease in KPH metastasis. Picrosirius red staining revealed that HIF-1α deletion has an unexpected effect on collagen organization (Fig. 1F, bottom). The collagen found in KPH tumors emits red birefringence, indicating higher levels of organization, whereas KP tumors contain collagen-emitting green birefringence, indicating that the collagen is more “immature.” Processed mature collagen emits red birefringence in normal tissues (34). However, collagen organization/maturity can be aberrant in tumors, as indicated by green birefringence (34). Furthermore, collagen organization has been shown to decrease (change birefringence gradually from red to green) as tumors worsen in stage and grade (34). Picrosirius staining of normal tissue (Supplementary Fig. S1B, white arrow) adjacent to KP tumors (black arrow) clearly shows the strong red/orange birefringence associated with normal collagen (blue arrow) in muscle tissue (white arrow). The lack of mature collagen organization in KP tumors is consistent with the idea that normal collagen modification and processing is disrupted because of elevated HIF-1α/PLOD2 activity.

Collectively, these findings suggest that the loss of HIF-1α alters collagen fiber deposition in primary sarcomas, and that PLOD2 may be a critical downstream target. To quantify the levels of PLOD2 in control and HIF-1α-deficient sarcomas, we derived cell lines from KP and KPH tumors. HIF-1α and PLOD2 were elevated in KP cells exposed to hypoxia (0.5% O₂) for 16 hours, whereas KPH cells did not express HIF-1α or PLOD2 under these conditions (Fig. 1G). A longer exposure of the Western blotting analyzing HIF-1α and PLOD2 expression at 21% O₂ clearly shows that both proteins are expressed under normoxic conditions (Supplementary Fig. S1C), but are dramatically upregulated in response to hypoxia. Finally, qRT-PCR showed that Pld2 mRNA levels were increased in hypoxic KP (KP1 and KP2) cells but not in KPH cells (KPH1 and KPH2, Fig. 1H). Therefore, Pld2 seems to be a HIF-1α-regulated target in KP tumors and cells.

To show that our results were not unique to a specific genetic background, we investigated tumor cell lines derived from a distinct mouse model of sarcoma, LSL-KrasG12D/+; Ink4a/Arf−/−; “KIA.” Sarcomas initiated by Adeno-Cre injection into KIA mice display similar growth kinetics and histopathology as in KP mice (8). We confirmed that PLOD2 is also a hypoxia-induced HIF-1α target in KIA cells (Fig. 1I). Quantification of three independent Western blot analyses showed that PLOD2 was significantly upregulated under hypoxia (P = 0.0118; data not shown). Deletion of HIF-1α using lentiviral-mediated short hairpin RNA (shRNA) significantly abrogated hypoxia-induced PLOD2 expression (P = 0.0008; data not shown). Similar results were obtained using the human fibrosarcoma cell line HT-1080 (Fig. 1J). Quantification of three independent Western blot analyses showed that PLOD2 was significantly upregulated under hypoxia (P = 0.0301; data not shown), and deletion of HIF-1α significantly abrogated hypoxia-induced PLOD2 expression (P = 0.0095; data not shown). qRT-PCR analyses of KIA (Fig. 1K) and HT-1080 (Fig. 1L) cells recapitulated these observations and showed that HIF-1α regulates PLOD2 at the mRNA level. We concluded that HIF-1α regulates PLOD2 expression in human and murine sarcoma cells and alters collagen deposition in primary murine sarcomas.

HIF-1α and PLOD2 Are Required for Metastasis in Sarcoma

To establish a role for the HIF-1α/PLOD2 pathway in sarcoma metastasis, we initially performed tail-vein injections using 1 × 10⁶ HT-1080 cells transduced with lentivirus-expressing scramble (Scr), HIF-1α, or PLOD2 shRNA (Supplementary Fig. S1D and S1E). Silencing either HIF-1α or PLOD2 significantly inhibited HT-1080 cell lung colonization, suggesting a role for this pathway in sarcoma pulmonary metastasis. To further explore potential contributions of HIF-1α to sarcomagenesis and metastases, we injected KIA (Supplementary Fig. S2A–S2C) and HT-1080 (Supplementary Fig. S2D and S2E) cells subcutaneously into nude mice. We observed no significant change in primary tumor volume or weight. Subsequently, we carried out similar experiments in KIA cells harboring PLOD2 shRNA for comparison to those treated with Scr or HIF-1α shRNA (Fig. 2A). Although the mean weight of PLOD2-deficient tumors was slightly higher than that of control or HIF-1α-deficient tumors (Fig. 2B), no differences in tumor volume were observed between groups (Fig. 2A), showing that HIF-1α and PLOD2 have little effect on primary tumor growth. However, silencing HIF-1α or PLOD2 caused a striking reduction in lung metastases in KIA-transplanted tumors (Fig. 2C–E), indicating that the HIF-1α/PLOD2 axis is necessary for pulmonary metastasis. HIF-1α ablation did not affect the expression of related HIF-1α targets, other than Pld2, including Lox, Serpine2, Col5a1, and Itgam in HIF-1α-depleted KIA tumors (data not shown) and cultured cells (Supplementary Fig. S2F). We confirmed that KIA tumors are hypoxic using Hypoxyprobe and HIF-1α staining. Serial sectioning of KIA tumors showed that hypoxic regions circumcribe more oxygenated cells surrounding blood vessels, which can be visualized by lectin staining (Fig. 2F, arrows). Importantly, picrosirius red staining of these tumor sections revealed that collagen is more organized in HIF-1α/PLOD2-deleted tumors (Fig. 2G), consistent with analyses of KP and KPH primary tumors (Fig. 1F). Similarly, deletion of HIF-1α and PLOD2 promoted the appearance of high-molecular weight collagen I dimer (**) and trimer (***) structures, based on Western blot analyses [Fig. 2H (16 hours) and Supplementary Fig. S2G (48 hours)]. Normal collagen production requires the assembly of individual α chain collagen molecules into organized and posttranslationally modified triple-helix structures (22). We have observed that KP sarcoma cells lack these trimmer structures, and dimer intermediates, in favor of monomeric collagen I. Deletion of the HIF-1α/PLOD2 pathway allows normal maturation to occur in these cells. Interestingly, a recent study examining mutations involved in Bruck syndrome and osteogenesis imperfecta has shown that biallelic mutations in PLOD2 promote collagen I trimer formation in patient-derived dermal fibroblasts (35). This finding is consistent with our observation that deletion of HIF-1α-mediated PLOD2 expression promotes the formation of higher-order collagen I structures. The changes we observed in collagen organization may be due to alterations in posttranslational modifications found on collagen. Deletion of HIF-1α and PLOD2 results in increased hydroxyproline levels in KIA tumors (Fig. 2I),...
HIF-1α and PLOD2 are key modulators of sarcoma metastasis

**Figure 2.** HIF-1α and PLOD2 are dispensable for primary sarcoma formation but essential for metastasis.

A, tumor allograft using 1 × 10⁶ Scr, HIF-1α-deficient, or PLOD2-deficient KIA cells subcutaneously injected into flanks of nude mice. n = 5 mice per group, two tumors per mouse; that is, 10 tumors per shRNA treatment. B, tumor weight was determined upon dissection of euthanized animals. PLOD2-deficient tumors were slightly larger than Scr tumors (P = 0.0495) and HIF-1α-deficient tumors (P = 0.0131). C, lungs from mice bearing KIA transplanted subcutaneous tumors. H&E staining revealed the presence of numerous metastases in control tumors (large purple areas) but very few in lungs from animals bearing PLOD2- and HIF-1α-deficient tumors. D, percentage of tumor burden was evaluated with ImagePro7 software; P = 0.39. E, loss of HIF-1α in the primary tumor significantly reduced the total number of sarcoma foci in lungs (P < 0.0001; left) calculated over multiple experiments and the average number of sarcoma foci/lung (P = 0.0500; middle). Loss of PLOD2 also significantly reduced the total number of sarcoma foci in lungs (P < 0.0001) and the average number of sarcoma foci/lung (P = 0.0453). HIF-1α and PLOD2 depletion in primary tumors also decreased the percentage of lungs with sarcoma foci: I, right, H&E, HIF-1α, Hypoxyprobe, and lectin staining were used to characterize control KIA tumors using a ×50 objective (scale bar, 200 μm) and a ×200 objective (scale bar, 100 μm). Black boxes indicate enlarged areas; arrow (left) indicates HIF-1α-positive cells. Arrow (right) indicates lectin-positive cells of a blood vessel. G, picrosirius red stain was used to characterize collagen organization in subcutaneous KIA tumors. Deletion of HIF-1α and PLOD2 altered collagen organization. Scale bar: 50 μm. H, 4% to 12% NuPage Bis-Tris (top) and 3% to 8% Tris–Acetate (middle) gradient gels were used to characterize collagen structure in KP cells treated with scr, HIF-1α, or PLOD2 shRNA: *monomers, **dimers, and ***trimers of collagen I were detected. Confirmation of PLOD2 and HIF-1α deletion was established using standard 10% SDS-PAGE gels (bottom). Hypoxia exposure lasted 16 hours. I, hydroxyproline modifications on collagen were measured using acid-hydrolyzed tumor tissue. Deletion of HIF-1α (P = 0.0536) and PLOD2 (P = 0.0102) increased hydroxyproline levels compared with Scr control. H&E, hematoxylin and eosin, GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
indicating that a high level of PLOD2 activity, resulting in elevated lysine hydroxylation, suppresses proline hydroxylation and mature “normal” collagen organization. Therefore, HIF-1α/PLOD2 deletion allows for increased prolyl hydroxylation, stabilizing mature collagen organization. On the basis of these data, we conclude that HIF-1α-mediated PLOD2 expression is not essential for primary sarcoma tumor growth, but is required for efficient lung metastasis through effects on collagen maturation.

**HIF-1α and PLOD2 Specifically Control Sarcoma Cell Migration**

HIF-1α and collagen deposition are known to promote metastasis by regulating tumor cell migration and invasion (13, 15, 30, 36, 37). We hypothesized that HIF-1α might regulate these processes in sarcomas through upregulation of PLOD2 transcription. Boyden chamber–based migration assays, using immunofluorescent staining of migratory cell nuclei with 4′,6-diamidino-2-phenylindole (DAPI), showed that shRNA-mediated knockdown of HIF-1α and PLOD2 significantly decreased sarcoma cell motility under hypoxia in KP (Supplementary Fig. S3A), KIA (Supplementary Fig. S3B), and HT-1080 cells (Supplementary Fig. S3C). The HIF-1α and migration findings are consistent with data presented by Kim and colleagues (38). Loss of KP cell motility due to HIF-1α and PLOD2 deletion under normoxic conditions (Supplementary Fig. S3A) can be attributed to the presence of both proteins at 21% O2 (Supplementary Fig. S1C).

It is well established that HIF-1α can influence cell migration by modulating multiple cell-intrinsic effectors, including the expression of Snail and Twist1 (36, 37, 39). We reasoned that if altering extracellular collagen deposition was the primary effect of HIF-1α on sarcoma cell migration, then the presence of wild-type sarcoma cells should rescue defects in matching HIF-1α–deficient cells in *in vitro* migration assays. To test this directly, we conducted scratch migration assays using stable Scr, HIF-1α–deficient, and PLOD2-deficient cells transduced with lentivirus bearing a dsRed expression vector (Scr shRNA) or a copepod GFP (copGFP) expression vector (HIF-1α shRNA, PLOD2 shRNA). Migration of HIF-1α–deficient copGFP+ cells and PLOD2-deficient copGFP+ cells was significantly delayed compared with control Scr dsRed cells in the KIA (Fig. 3A and B), KP (Supplementary Fig. S4A and S4B), and HT-1080 cell lines (Supplementary Fig. S5A and S5B).

**Figure 3.** HIF-1α and PLOD2 mediate sarcoma cell migration via a cell-extrinsic mechanism. **A**, scratch migration assays of confluent, and therefore oxygen- and nutrient-limited, KIA cells stably expressing Scr, HIF-1α– or PLOD2-specific shRNAs and either copGFP (HIF-1α, PLOD2) or dsRed (Scr). Green and red cells were mixed 1:1. **B**, quantification of recovery in A; all P values are ≤ 0.0105. **C**, Western blot analyses of KIA cells treated as in A and B. ShRNA-mediated knockdown of HIF-1α and PLOD2 shown here also reflects knockdown occurring in A and B as cell lines generated for these assays were then transduced with copGFP lentivirus of dsRed lentivirus. **D**, proliferation of KIA cells expressing Scr, HIF-1α– or PLOD2-specific shRNAs under hypoxic conditions. Cells were counted daily.

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and PLOD2 are key modulators of sarcoma metastasis.

**Figure 4.** PLOD2 expression rescues sarcoma migration but not invasion. A, quantification of migration assays of normoxic and hypoxic KIA cells expressing Scr- or HIF-1α-specific shRNAs and ectopically expressing control or wild-type human PLOD2 cDNA. All P values are ≤0.0432. B, quantification of HT-1080 migration assays conducted as in A, except murine Plod2 was ectopically expressed. All P values are ≤0.0431. C, representative images of Boyden chamber migration assay using HT-1080 cells treated as in B, scale bar, 50 μm. D, quantification of invasion assays of normoxic and hypoxic KIA cells expressing Scr- or HIF-1α-specific shRNAs and ectopically expressing control or wild-type human PLOD2 cDNA using Matrigel-coated Transwell invasion chambers. All P values are ≤0.0084. E, quantification of HT-1080 invasion assays conducted as in D, except murine Plod2 was ectopically expressed. All P values are ≤0.0017. F, representative images of Matrigel-coated chamber invasion assay using KIA cells treated as in D; scale bar, 50 μm.

...and SSβ). However, when control and knockdown cells were mixed together, migration of both HIF-1α- and PLOD2-deficient cells was restored to control levels (Fig. 3A). Expression of copGFP and dsRed did not affect the ability of HIF-1α to modulate PLOD2 levels in any of the three cell types (Fig. 3C; Supplementary Figs. S4C and S5C). Importantly, deletion of HIF-1α and PLOD2 did not affect proliferation in these cell lines (Fig. 3D and Supplementary Fig. S5D). These data indicate that HIF-1α drives sarcoma cell migration in a cell-extrinsic manner, possibly through PLOD2-mediated effects on collagen modification.

To determine whether HIF-1α-mediated migration and invasion were PLOD2-dependent, we ectopically expressed PLOD2 in HIF-1α-deficient KIA (Fig. 4A) and HT-1080 cells (Fig. 4B and C). HIF-1α-deficient HT-1080 and KIA cells were transduced with control lentivirus or lentivirus bearing a wild-type PLOD2 expression vector. Western blot analysis showed endogenous and exogenous PLOD2 levels, as well as the efficacy of HIF-1α inhibition (Supplementary Fig. S6A). Murine Plod2 was expressed in human HT-1080 cells, and human PLOD2 in murine KIA cells, making it possible to evaluate changes in endogenous and ectopic PLOD2 mRNA levels by qRT-PCR using species-specific primers (Supplementary Fig. S6B). PLOD2 expression rescued cell migration in HIF-1α-deficient cells under hypoxic conditions and also stimulated migration in normoxic cells (Fig. 4A–C). Interestingly, PLOD2...
PLOD2 Lysyl Hydroxylase Activity Is Required for Sarcoma Cell Migration

PLOD2 lysyl hydroxylase activity is dependent upon association with several essential cofactors, including Fe**(ii)** and 2-oxoglutarate, which requires a conserved aspartate residue (D689 in human PLOD2; D668 in mouse), and mutation of these amino acids inactivates PLOD2 (27, 28). Using site-directed mutagenesis, we generated inactive PLOD2 (D689A and D668A) to determine whether the enzymatic activity of PLOD2 was essential for its ability to rescue cell migration in HIF-1α-deficient cells. We conducted migration assays on stable Scrl control and HIF-1α-deficient HT-1080 and KIA cells that had also been transduced with lentivirus bearing a mutant PLOD2 expression vector. We observed that expression of inactive PLOD2 mutants failed to rescue migration in HIF-1α-deficient KIA (Fig. 5A) and HT-1080 cells (Fig. 5B). Furthermore, mutant PLOD2 behaves as a dominant negative in KIA and HT-1080 cells, suppressing hypoxia-induced migration. Interestingly, significant overexpression of mutant PLOD2 modestly inhibited endogenous PLOD2 and HIF-1α levels as shown by qRT-PCR (Supplementary Fig. S6C and S6D) and Western blot analysis ( Supplementary Fig. S6E).

To confirm that PLOD2 is required for sarcoma cell migration, we used a previously described pharmacologic inhibitor of PLOD2 expression, minoxidil (40). Minoxidil treatment (0.5 mmol/L) for 12 hours significantly reduced HT-1080 cell migration (Fig. 5C and D), concomitant with reduced PLOD2 protein levels as shown by Western blot analysis of HT-1080 and KIA cells (Fig. 5E). Interestingly, minoxidil increased HIF-1α levels, but not cell migration, supporting the conclusion that HIF-1α-dependent induction of PLOD2 lysyl hydroxylase activity is required for sarcoma cell migration. To determine the physiologic importance of minoxidil as a sarcoma metastasis inhibitor, we generated allografts of subcutaneously injected KIA cells in nude mice and immediately began injections of PBS, 1 mg/kg minoxidil, or 3 mg/kg minoxidil every other day for 3 weeks. Minoxidil had no effect on primary tumor volume, tumor weight, or overall animal health/weight (Fig. 5F and Supplementary Fig. S7A and S7B). However, minoxidil treatment significantly reduced the number of pulmonary metastases (Fig. 5G and S7B). Consistent with our observations in the autochthonous model, minoxidil-treated tumors contained relatively organellar collagen compared with control-treated tumors, based on picrosirius red birefringence (Fig. 5I). These data show the potential usefulness of minoxidil as a treatment for premetastatic sarcoma.

In Vivo Metastasis Requires HIF-1α/PLOD2-Mediated Collagen Production

We tested the hypothesis that HIF-1α-dependent PLOD2 expression and collagen modification are required for cell migration and metastasis in vivo using the KIA tumor transplant model of metastatic UPS. Staining of HIF-1α-deficient tumor sections with Masson’s Trichrome revealed a significant change in collagen staining, confirming that collagen was altered in the absence of HIF-1α (Fig. 6A and B). Collagen was quantified using ImagePro7 software, which revealed a dramatic shift in collagen density in HIF-1α-deficient tumors compared with control tumors (Fig. 6C). Intriguingly, HIF-1α-deficient tumor cells appear smaller and rounder than control cells, which may reflect their relative inability to associate with and migrate along collagen fibers. To investigate this possibility, we conducted second harmonic generation (SHG) analysis of explanted control and HIF-1α-deficient tumors (Fig. 6A and B). SHG imaging permits the covisualization of endogenous collagen (red) and GFP+ tumor cells (green) in live tissue. In control tumors, we identified areas of collagen deposition and observed that GFP+ tumor cells associated closely with collagen and their morphology was elongated as they adhered to collagen fibers. In contrast, in HIF-1α-deficient tumors, cells did not elongate or associate with collagen (Fig. 6A–C). Furthermore, HIF-1α deletion causes a fourfold decrease in percentage of collagen area (Fig. 6C) and a concomitant fourfold decrease in murine lung metastasis (Fig. 2D, middle). We conclude from these observations that there is a correlation between HIF-1α-mediated collagen modification and metastasis. PLOD2-deficient tumors also possess defects in collagen maturation and cellular morphology, phenocopying what is seen when HIF-1α is silenced (Fig. 6D). In many epithelial tumors, mesenchymal cells such as fibroblasts are recruited and subsequently secrete collagen. However, we reasoned that in mesenchymal lesions, the tumor cells themselves would likely perform this function. Therefore, we sought to determine whether a fibroblast population depositing additional collagen was present in our sarcoma model. Immunofluorescence analysis of GFP+ KIA tumor sections stained for GFP and the mesenchymal marker vimentin showed that a small percentage of cells in the tumor were GFP+ ‘vimentin’ as expected for an infiltrating fibroblast population (Supplementary Fig. S8A). We quantified this population by flow cytometry using single-cell suspensions of dissociated GFP+ tumors. Roughly 12% of cells in these tumors were GFP+ infiltrating cells (Supplementary Fig. S8B). These data suggest that the tumor cells themselves (and not additional stromal cells) deposit the majority of collagen secreted in sarcomas.

Perhaps the most important aspect of collagen-associated tumor cell migration is the ability of the collagen network to deliver cells to the vasculature. To determine whether this process is compromised in HIF-1α-deficient sarcomas, we quantified the instances where collagen invades blood vessels in the tumors. In the absence of HIF-1α, the loss of collagen at the blood vessels was severe (Fig. 6B). These findings are consistent with the overall conclusion that loss of HIF-1α prevents tumor cells from migrating to vessels and escaping the primary lesion. PLOD2 ablation results in defects in collagen/vessel association similar to that of HIF-1α-deficiency (Fig. 6D and D). Masson’s Trichrome staining of minoxidil-treated tumors showed results similar to those of HIF-1α- and PLOD2-deficient tumors (Fig. 6E). In HIF-1α/PLOD2-deficient tumors, deposited collagen appears thinner and does not penetrate the vasculature, preventing tumor cells from using the collagen “highway” to disseminate to distant sites.
HIF-1α and PLOD2 Are Key Modulators of Sarcoma Metastasis

**Figure 5.** PLOD2-mediated control of cell migration and metastasis is dependent upon its lysyl hydroxylase activity. 

A, quantification of migration assay of normoxic and hypoxic KIA cells expressing Scr- or HIF-1α-specific shRNAs and ectopically expressing control or mutant human PLOD2 D668A cDNA (all P values < 0.0001). B, quantification of migration assay of normoxic and hypoxic HT-1080 cells expressing Scr- or HIF-1α-specific shRNAs and ectopically expressing control or mutant murine Plod2 D668A cDNA (all P values ≤ 0.0010). C, scratch migration assays of HT-1080 and KIA cells stably expressing copGFP in the presence or absence of 0.5 mmol/L minoxidil pretreatment for 48 hours. D, quantification of recovery from C; P ≤ 0.0058. E, Western blot analyses of HT-1080 and KIA cells treated as in C and D; F, tumor allograft growth using 1 × 10⁶ KIA cells subcutaneously injected into flanks of nude mice; n = 10 mice per group, two tumors per mouse; that is, 20 tumors per treatment with vehicle or minoxidil. G, lungs from mice bearing KIA-transplanted subcutaneous tumors treated with PBS or minoxidil. H&E staining revealed the presence of numerous metastases in control tumors (large purple areas) but very few in lungs from animals treated with minoxidil. H, intraperitoneal minoxidil treatment reduced the average number of sarcoma foci/lung. I, picrosirius red staining of KIA subcutaneous tumors from F. Minoxidil treatment altered collagen organization in the primary tumors; scale bar, 50 μm. H&E, hematoxylin and eosin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
PLOD2 Expression Rescues *In Vivo* Metastasis of HIF-1α–Deficient Primary Sarcomas

To clearly show that HIF-1α–mediated regulation of PLOD2 is essential for metastasis, we carried out an *in vivo* rescue experiment in which wild-type PLOD2 was ectopically expressed in HIF-1α–deficient tumors (Fig. 7A–C). Expression of PLOD2 markedly rescued metastatic potential in KIA tumors (Fig. 7B and C), while having no reproducible effect on primary tumor volume (Fig. 7A). Given that PLOD2 promotes *in vivo* metastasis (Fig. 7A and B) and is required for lung colonization in a tail vein injection assay (Supplementary Fig. S1D and S1E), we surmised that PLOD2 likely plays a critical role during *in vivo* blood vessel intravasation and extravasation, but this function is not assayable using...
and PLOD2 are key modulators of sarcoma metastasis

HIF-1α and PLOD2 are key modulators of sarcoma metastasis (Fig. 3D and Supplementary Fig. S5D). Thus, we conclude that HIF-1α expression is key to proliferation in either cell type over a 3-day period (Fig. 3D and Supplementary Fig. S5D). Thus, we conclude that HIF-1α and PLOD2 regulate multiple aspects of metastasis (migration, attachment to collagen networks, and vessel invasion).

DISCUSSION

Soft-tissue sarcomas are a highly complex set of malignancies, comprising more than 50 histologically distinct subtypes associated with genetic alterations in diverse molecular pathways (5). Although lethal metastases, particularly to the lung, are a common occurrence in patients with sarcoma, the molecular mechanisms regulating this process are largely unknown. Primary sarcomas are noted for extensive fibrosis and deposition of ECM components, a feature that has been associated with metastatic potential in numerous cancers (41, 42). Although many details are incompletely understood, it is clear that metastatic tumor cells can associate physically with dense collagen networks in solid tumors, and migrate along this collagen “highway” toward vascular tissues (18, 43), through which they ultimately disseminate and colonize distant organs. Therefore, therapeutic manipulation of collagen modification/organization in primary sarcomas, as well as other tumors, could have significant impact on an early, proximal step in metastasis, which remains the leading cause of cancer-related death.

Low intratumoral O2 levels and HIF-1α expression are key predictors of metastatic potential in sarcomas. Although previous microarray analyses revealed elevated HIF-1α and PLOD2 expression in sarcoma patient samples (32), the mechanisms by which these genes regulate sarcoma progression and/or metastasis were unclear. In this study, we showed that HIF1A and PLOD2 expression levels are preferentially elevated in primary human sarcomas that subsequently metastasized, suggesting that they regulate one or more aspects of tumor cell dissemination. Using independent murine sarcoma models, we determined that HIF-1α-dependent PLOD2 expression is...
required for deposition of the disorganized collagen required to facilitate tumor cell migration in primary tumors, as well as pulmonary metastasis. In vitro assays revealed that the HIF-1α/PLOD2 pathway regulates sarcoma cell migration in a collagen-dependent manner. Finally, ectopic expression of PLOD2 rescues both cell migration and metastatic potential in HIF-1α-deficient cells and tumors. Collectively, our data indicated that HIF-1α-dependent PLOD2 expression is essential for hypoxia-mediated collagen disorganization and metastasis in sarcomas (see model in Fig. 7D). Unexpectedly, we concluded that disorganized/immature collagen take on a denser structure and are alternately modified in a way that supports tumor cell adherence and migration. Deletion of HIF-1α/PLOD2 restores collagen modification and organization, preventing its association with tumor cells.

It is important to note that HIF-1α regulates multiple distinct pathways associated with metastasis in various tumor models. For example, HIF-1α reduces E-cadherin expression and promotes invasiveness and the epithelial-to-mesenchymal transition in renal cancers (44). Moreover, HIF-1α enhances metastasis by regulating TWIST1 transcription in head and neck squamous cell cancers (36, 37). However, in our sarcoma models, Twist1 and Snai1 expression were found to be insensitive to hypoxic stimulation (data not shown), making them less likely to contribute to hypoxia-mediated migration and invasion. HIF-1α has also been shown to play important cell-extrinsic roles in breast cancer models, where it induces expression of ECM-modifying enzymes, which promotes effective metastasis by modifying the premetastatic niche (13, 31). In addition, HIF-1α-dependent expression of prolyl hydroxylases as well as angiopoietin-like 4 and LiCAM promotes breast cancer metastasis by controlling tumor cell invasion of the vasculature (25, 45). In primary sarcomas, HIF-1α-dependent PLOD2 expression has a profound effect on extracellular collagen networks, which in turn regulate tumor cell migration and the initiation of metastasis. Interestingly, PLOD2 has been recently identified as a novel prognostic factor in hepatocellular carcinoma, in which it is associated with disease recurrence and intrahepatic metastases (46). Although previous work from multiple groups has highlighted the importance of collagen as a scaffold that supports the migration of metastatic tumor cells (18, 43), the cellular processes that create these collagen deposits are relatively understudied.

As hypoxia and HIF expression are important prognostic indicators in many solid tumors, conclusions drawn from the current studies may be applicable in multiple tumor contexts, although other collagen-modifying enzymes, such as PLOD1 or LOX, may also contribute in other tumor types. In sarcoma, further investigation of PLOD2 as a feasible therapeutic target is clearly warranted. We have used the PLOD inhibitor minoxidil to address the importance of PLOD2 in tumor cell migration and in vivo pulmonary metastases, although the usefulness of this drug in the clinic or as a lead compound for novel drug discovery remains to be determined.

Although metastases are responsible for the vast majority of cancer-associated deaths, there are very few therapeutic approaches that specifically target metastasis in any tumor model. On the basis of our findings, it seems prudent to pursue clinical agents that would inhibit metastasis by promoting the ECM network in conjunction with the current standard of therapy.

**METHODS**

**Gene Expression Analysis**

RNA was isolated from human tumor tissue using the RNeasy Kit (Qiagen), and quality was analyzed using a 2100 Bioanalyzer (Agilent Technologies). Amplification was achieved using the Total-Prep RNA Amplification Kit (Illumina). Amplified cRNAs were then hybridized on HumanRef8 Expression Beadchips (Illumina), which target more than 24,000 genes. Image analysis was conducted using Illumina’s BeadStudio v3.0.14 Gene Expression Module. All statistical analyses were conducted using the statistical software R (http://www.r-project.org). Supervised hierarchical clustering of 140 genes transcriptionally regulated by HIF-1α was conducted using 1-r (Pearson correlation) as a distance metric with a complete linkage. Data from this analysis were used to determine relative gene enrichment.

**Mouse Models**

Generation of Hif1αfl/fl (47) and KP mice has been previously described (8). These mice were crossed to create the KPH animals. Soft-tissue sarcomas were generated by intramuscular injection of a calcium phosphate precipitate of Ad-Cre (Gene Transfer Vector Core, University of Iowa, Iowa City, IA). For transplant tumors, 1 x 10^6 KIA cells (derived from KIA tumors) were injected subcutaneously into the flanks of nu/nu mice (Charles River Laboratories). In each experiment, 10 mice per experimental group were used with each mouse bearing two subcutaneous tumors. Tumors developed after 3 to 5 days and were monitored every other day, and animals were euthanized after 20 to 30 days. For in vivo lung metastasis analysis, lung tissues were removed and sections were stained with hematoxylin and eosin. Images were acquired using a Nikon SMZ2800 stereoscope with a Nikon 1200f digital camera and Nikon Act-1 software. The ratio of total metastatic sarcoma foci to total lung area (% tumor burden) was determined using ImagePro 6.5 software (Media Cybernetics Inc.). In addition, the percentage of animals in the entire cohort with metastatic foci was evaluated along with the total number of metastatic foci per experiment and the average number of sarcoma lung foci per lung in each experiment. For minoxidil injections, allografts and drug treatments were started simultaneously. Mice bearing subcutaneous KIA tumors on each flank were intraperitoneally injected with PBS, 1 mg/kg minoxidil, or 3 mg/kg minoxidil every other day unless the animals were euthanized, and the tumors and lungs were removed.

**Cell Culture, Treatment, and Lentiviral Transduction**

HT-1080 cells were purchased from the American Type Culture Collection on January 8, 2010. The cells were authenticated by karyotyping and DNA profiling from the initial seed stock. KIA cells were derived in the laboratory from KIA tumors, as described elsewhere (8, 48). Finally, KP1 and KP2 were derived from KP tumors and KPH1 and KPH2 cells were derived from KPH tumors. Low-oxygen conditions were achieved in a Ruskinn in vivO2 400 work station. Cells were treated with 0.5 mmol/L minoxidil diluted in Dulbecco’s Modified Eagle Medium (DMEM) culture media (Sigma-Aldrich) for 36 to 48 hours, and the drug was replenished every 24 hours. For shRNA-mediated knockdown of Hif1a, Hif1a, Plod2, and PLOD2, lentiviral particles bearing pLKO.1 shRNA plasmids were generated in HEK-293T cells. 293T cells were transfected overnight with pLKO.1 empty-vector, nonspecific shRNA, or target-specific shRNA and viral packaging plasmids, according to the Fugene reagent protocol (Roche). The following shRNA pLKO.1 plasmids were used: pLKO.1 Scr shRNA (Addgene 1864), pLKO.1 Hif1a shRNA...
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Immunostaining and Imaging

Immunohistochemistry of tissue sections with antibodies to HIF-1α (Abcam) and lectin (Vector Laboratories) was conducted using enzymatic Avidin–Biotin Complex (ABC; 3,3'-diaminobenzidine (DAB) staining (Vector Laboratories). Nuclei were counterstained with hematoxylin. Immunofluorescence staining of copGFP (Evrogen) and vimentin (Abcam) as well as DAPI (Invitrogen)-stained images were visualized using an Olympus IX81 microscope. Collagen was stained using the Mason’s Trichrome Kit (Sigma-Aldrich), and nuclei were counterstained with Weigert’s iron hematoxylin (Sigma-Aldrich). Collagen SHG images were captured using a Prairie Technologies Ultima 2-Photon Microscope system. Images were taken with an excitation wavelength of 457–487 nm (that detects the SHG signal for collagen). Collagen was quantified using Image-Pro software. All enzymatic Avidin–Biotin Complex (ABC)-3,3′-diaminobenzidine (DAB) staining (Vector Laboratories). copGFP is particularly bright and thus is suitable for in vivo SHG studies as well as cell culture. dsRed was expressed from the pULTRA-HOT vector (Addgene plasmid #24130) originally constructed by Didier Trono.

Western Blotting, qRT-PCR, and Hydroxyproline Measurement

Whole-cell lysates were prepared in SDS/Trits pH 7.6 lysis buffer. Proteins were electrophoresed and separated by SDS-PAGE and transferred to nitrocellulose membranes and probed with the following antibodies: rabbit anti-HIF-1α (Cayman Chemical Co.), rabbit anti-collagen I (Millipore), and rabbit anti-PLOD2 (Proteintech). Densitometry was conducted using ImageJ software. Representative Western blot analyses from multiple independent experiments are presented. Total RNA was isolated from cells using the TRIzol reagent protocol (Invitrogen) and from tumor tissue using the RNeasy Mini Kit (Qiagen). mRNA was reverse transcribed using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems). Transcript expression was determined by quantitative PCR of synthesized cDNA using the Applied Biosystems 7900HT system. Target cDNA amplification was measured using TaqMan primer/probe sets (Applied Biosystems) for human and murine HIF1A, PLOD2, HPRT1 (control), Lac, Serpin2, Col5a1, and Ilv3. Hydroxyproline was assessed using homogenized tumor samples and the Hydroxyproline Assay Kit (Biovision Inc.).

Migration, Invasion, and Proliferation Assays

Migration assays were conducted using 24-well chambers with inserts (8-µm pores; BD Biosciences). Medium containing 10% serum was placed in the lower chamber, and tumor cells (1 × 10⁵) suspended in medium without serum were added to the top chamber. The plates were incubated under 21% or 0.5% O₂ for 4 (HT-1080) or 18 hours (KIA). After migration, nonmigratory cells were removed from the top of the insert membrane using cotton swabs. The underside of each membrane was fixed in methanol and stained with DAPI (Invitrogen), and the number of cells that migrated completely through the 8-µm pore was determined in six random high-power fields (×20 objective) for each membrane. Invasion was examined in a similar way using Matrigel-coated inserts (BD Biosciences). Scratch assays were conducted on confluent KP, KIA, and HT-1080 cells expressing either dsRed or copGFP and seeded onto plates at a 1:1 ratio. Cells were imaged under normoxic conditions. ImageJ software was used to measure areas devoid of cells in five unique fields per condition. As cells migrated into the wounds, those areas became smaller. We determined the average area lacking GFP+-cells per condition and displayed those means as a normalized percentage with the prewounding image representing the baseline, and then generated recovery statistics. Proliferation was assessed by counting cell numbers manually using a hemocytometer every day for 4 days.

Flow Cytometry

Tumors were dissected, homogenized, and collagenase-treated to generate a single-cell suspension. Live cells were run on a BD LSR II flow cytometer for the detection of GFP. GFP- parent cell lines were also run to set up GFP- and GFP+ gates.

Statistical Analysis

Data are represented as mean ± SEM. Unpaired two-tailed Student t test was conducted for most of the studies to evaluate the differences between the control and experimental groups. P ≤ 0.05 was considered statistically significant. Significance is indicated by the presence of an asterisk “*.” Quantified data shown represent at least three independent experiments. GraphPad Prism software was used to conduct all statistical analyses.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: T.S.K. Eisinger-Mathason, E. Puré, S. Yoon, D.G. Kirsch, M.C. Simon


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T.S.K. Eisinger-Mathason, M. Zhang, Q. Qiu, N. Skuli, M.S. Nakazawa, T. Karakashova, V. Mucay, J.E.S. Shay, L. Stangenberg, N. Sadri, M.C. Simon

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T.S.K. Eisinger-Mathason, M. Zhang, Q. Qiu, E. Puré, D.G. Kirsch, M.C. Simon

Writing, review, and/or revision of the manuscript: T.S.K. Eisinger-Mathason, M. Zhang, Q. Qiu, E. Puré, S. Yoon, D.G. Kirsch, M.C. Simon

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N. Skuli, J.E.S. Shay

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Hypoxia-Dependent Modification of Collagen Networks Promotes Sarcoma Metastasis

T.S. Karin Eisinger-Mathason, Minsi Zhang, Qiong Qiu, et al.

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