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Original Article

Increased expression of collagen prolyl hydroxylases in ovarian cancer is associated with cancer growth and metastasis

Mihae Song[1], Erica Schnettler[2,4], Annapoorna Venkatachalam[3,4], Yujun Wang[5], Lisa Feldman[5], Peter Argenta[6], Lorna Rodriguez-Rodriguez[1], Sundaram Ramakrishnan[7]

[1]Division of Gynecologic Oncology, Department of Surgery, City of Hope National Medical Center, Duarte, CA, USA; [2]Oncology Medical Affairs, Illumina, Greater Minneapolis-St. Paul, Minneapolis, MN, USA; [3]Mayo Clinic, Rochester, MN, USA; [4]Department of Pharmacology, University of Minnesota, Minneapolis, MN, USA; [5]Division of Neurosurgery, Department of Surgery, City of Hope National Medical Center, Duarte, CA, USA; [6]Department of Obstetrics, Gynecology and Women's Health, University of Minnesota, Minneapolis, MN, USA; [7]Department of Surgery, University of Miami, Miami, FL, USA

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Abstract: Tumor hypoxia induces collagen deposition and extensive extracellular matrix remodeling, significantly enhancing the processes of invasion and metastasis. Collagen prolyl-4-hydroxylases (P4HA) play a critical role in collagen post-translational modification. The primary objective of this study is to comprehensively assess the role of P4HA in promoting ovarian cancer growth and facilitating metastasis. Human epithelial ovarian cancer cells were transfected with shRNAs to target P4HA1 and P4HA2. The impact of P4HA knockdown on crucial factors such as collagen I deposition, cell proliferation, and migration were examined *in vitro*. Additionally, *in vivo* studies involved the injection of both control and P4HA knockdown cells into athymic mice, enabling the assessment of tumor growth and peritoneal metastasis. The relevance of prolyl hydroxylases to clinical outcomes was then determined by analyzing clinical databases. Quantitative RT-PCR showed upregulation of P4HA1 and P4HA2 mRNA in A2780 cells when exposed to hypoxia. ShRNA-mediated downregulation of P4HA1 and P4HA2 significantly reduced the deposition of collagen I. Knockdown of P4HA expression reduced cell proliferation *in vitro* and peritoneal seeding *in vivo*. A2780 cells stably transfected with shP4HA1 and shP4HA2 inhibited tumor growth and metastases in athymic mice. Furthermore, our review of the TCGA dataset revealed that increased P4HA1 and P4HA2 mRNA levels are associated with decreased overall survival in patients with ovarian cancer. The increased expression of collagen P4HA has been linked to ovarian cancer growth and metastasis. This evidence highlights their potential as prognostic biomarkers and promising therapeutic targets.

Keywords: Collagen prolyl hydroxylases (P4HA), ovarian cancer, metastasis, tumor hypoxia, extracellular matrix

Introduction

Ovarian cancer stands as the leading cause of gynecologic cancer-related fatalities in the United States [1]. Despite initial aggressive surgery and chemotherapy, over 75% of patients with epithelial ovarian cancer (EOC) relapse after standard-of-care therapy and die from the disease, with a 5-year survival rate of 40% [1, 2]. Most women diagnosed with EOC present at advanced stages due to the lack of pathognomonic symptoms and effective screening tools. Metastasis is accountable for approximately 90% of all cancer-associated deaths [3]. Understanding how ovarian cancer spreads is essential for developing new and effective treatments that improve the quality of life for patients with EOC.

Metastatic progression in EOC involves interactions between tumor cells and the extracellular matrix (ECM), where ECM remodeling promotes cancer growth, immune infiltration, and metastasis. The ECM in EOC comprises approximately 300 proteins that regulate tissue homeostasis, inflammation, and disease, of which about 90%

is collagen [4-7]. The large collagen family has unique structural domains, forming a triple-helical structure of three alpha chains. The alpha chains have a characteristic motif with glycine located at every third amino acid residue and the presence of post-translationally modified hydroxyl prolines [8]. Most of the proline modifications in collagen result in 4-hydroxyproline, whereas a small fraction of 3-hydroxyproline is also observed. The modification of 4-hydroxyproline is considered to be the most significant, as it provides thermal stability and facilitates the formation of triple helical configurations in collagen fibrils at physiological temperatures.

Prolyl 4-hydroxylase is the key enzyme responsible for proline hydroxylation in collagens. It is made of two alpha and two beta chains. The alpha chain is enzymatic, and the beta chain provides structural stability and has a protein disulfide isomerase function. There are three isoforms of alpha polypeptide constituting the enzymes (P4HA1, P4HA2, and P4HA3) [9]. Post-translational modifications of procollagen by P4HA is essential for the proper three-dimensional folding of the newly synthesized procollagen chain and deposition [10]. Increased collagen accumulation can activate this dynamic process, which changes the tumor microenvironment's physical and biochemical properties and regulates cancer cell migration and signaling [11, 12]. Collagens interact with integrins, ECM components such as fibronectin, and cellular tyrosine kinase receptors such as discoidin domain receptors (DDR1 and DDR2). These complex interactions modulate cell adhesion, spreading, motility, and invasion.

Tumor hypoxia stimulates ECM remodeling through hypoxia-inducible factor (HIF-1) alpha, which activates many enzymes of the collagen biosynthesis pathway, including P4HA1 and P4HA2 [13]. Several studies have shown that increased deposition of collagen 1 contributes to tumor migration and metastasis [14]. P4HA2 was shown to be a key regulator in several gene signatures for hypoxia, epithelial to mesenchymal transition (EMT), and a hallmark of metastasis and migration [15].

Several studies have suggested that P4HA1 and P4HA2 are upregulated and essential for metastasis in many cancers, including breast and prostate [16-18]. Evaluation of a panel of nine hypoxia-regulated genes showed upregulation of P4HA1 under hypoxic conditions when compared to normoxic conditions in human ovarian cancer cell lines and xenograft tumor tissues [19]. P4HA2 was also markedly upregulated in EOC clinical samples, and high expression of P4HA2 was associated with poor clinical outcomes [20]. However, the role of P4HA in ovarian cancer metastasis has not been fully elucidated. Our study aimed to explore and evaluate role of P4HA in the remodeling the microenvironment in EOC and its impact on metastases.

Materials and methods

Cell culture and sh-RNA mediated knockdown

Human ovarian cancer cells (A2780, obtained from Thomas Hamilton, Fox Chase Cancer Center, Philadelphia) were cultured in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 1% glutamine, 1% Penicillin-Streptomycin, and 1% sodium pyruvate. Cells were transfected with scrambled RNA sh-control vector (pGIPZ), sh-P4HA1 (Clone # 131297, Open Biosystem, Huntsville, AL, United States), sh-P4HA2 (Clone # 33554, Open Biosystem), or a combination of sh-P4HA1 and sh-P4HA2 (sh-P4HA1/2) using lipofectamine (Invitrogen, Waltham, MA, United States). Transfected cells were selected for puromycin resistance for three weeks.

Hypoxia-induced changes

A2780 and OVCAR 8 cells were seeded and cultured overnight before exposure to normoxia (21% O_2) or hypoxia (1% O_2). To achieve hypoxia, dishes containing cells with about 80% confluency were placed in a modular chamber (Billups Rothenberg, Inc., Del Mar, CA, United States) and flushed with a mixture of 1% O_2, 5% CO_2, and 94% N_2 at 10 L/min for 15 minutes, per previously published methods [21]. One hour later, the chamber was flushed again for 10 minutes. Chambers remained tightly sealed and placed in a 37°C incubator for 6 or 24 hours. For reoxygenation, 24-hour hypoxic-treated dishes were placed in a standard culture incubator for another 6 and/or 24 hours and hyperoxia (95% O_2) for another 6 hours. Partial treated cells were collected to isolate total RNA using TRIzol™ Reagent (cat#: 15596026, TheroFisher Scientific, MA, USA), and relative transcript levels were analyzed

using qRT PCR. Partially treated cells were collected for protein lysate using cell lysis buffers (cat#: FNN0011, TheroFisher Scientific, MA, USA) for western blotting. The GAPDH gene was used as a reference gene. Vascular endothelial growth factor (VEGF) was used as a positive control to validate hypoxia-induced transcriptional upregulation.

Western blot analysis

A2780 cells were cultured until 80% confluent, then lysed with 60 μL RIPA buffer (Alfa Aesar, Haverhill, MA, United States) containing protease and phosphatase inhibitor cocktail (Thermo Scientific, Waltham, MA, United States). Protein concentration was measured using BCA™ Protein Assay Kit (Pierce Biotechnology, Waltham, MA, United States) and 25 μg of lysates were resolved in 12% SDS-PAGE gels and transferred to a nitrocellulose membrane (BIO-RAD, Hercules, CA, United States). Blots were blocked with 5% non-fat milk for one hour at room temperature. Membranes were then incubated with primary antibody (rabbit polyclonal antibodies to human P4HA1 and P4HA2, Abcam, Cambridge, MA, United States) overnight at 4°C and counterstained with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:2000) at room temperature for one hour. Immunoblots were developed with Clarity™ Western ECL Substrate (BIO-RAD) using an enhanced chemiluminescence method. Densitometric analyses and quantification of bands were carried out using ImageJ software, per the manufacturer's protocol.

Enzyme-linked immunosorbent assay (ELISA)

Collage type 1 secreted into cell supernatants was measured by an enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MI, United States), according to the manufacturer's instructions. Each experimental measurement was conducted in triplicates to ensure reliability, and the data presented indicate the mean value derived from three independent experiments.

Real-time cell proliferation

A2780 cells transfected with either the sh-control vector, sh-P4HA1, sh-P4HA2, or sh-P4HA1/2 were seeded into E-Plate 16 (ACEA BioSciences, San Diego, CA, United States). Real-time cell proliferation was determined by electrical impedance measurements over a period of 48 hours using the xCelligence system (Agilent, Santa Clara, CA, United States) per previously published methods [22].

Cell migration (scratch wound assay)

A2780 cells transfected with either the sh-control vector, sh-P4HA1, sh-P4HA2, or sh-P4HA1/2 was seeded in 6-well plates and allowed to attach for 24 hours. A scratch wound was made using a 1000 μl sterile pipette tip, as previously described, and continued to culture for 24 hours [23]. Cell migration (the extent of wound closure) was monitored under microscopy, and images were captured at 0 and 24 hours. The closure of the scratch wound area was measured using ImageJ software. Each experiment was carried out in triplicate, and the data represented values from three independent experiments.

Effect of P4HA down regulation on peritoneal seeding and growth of ovarian cancer cells

All animal studies were carried out with approval from the Institutional Animal Care and Use Committee (IACUC). Female, 6-8-week-old, athymic mice were injected intraperitoneally with 5×10^6 A2780 sh-control or sh-P4HA1/2 cells, with seven mice in each group. shRNA constructs (pGIPZ) co-express green fluorescent protein (GFP). Animals were sacrificed after seven days. The mice were imaged at 4× magnification using a Nikon AZ100M fluorescence microscope. At the experimental endpoint, tumor nodules were counted, harvested, weighed, and fixed in buffered formalin for sectioning.

The impact of P4HA knockdown on the growth of ovarian cancer cells was assessed by injecting A2780 cells transfected with sh-control, sh-P4HA1, sh-P4HA2, or sh-P4HA1/2 into twelve mice in each group.

Second harmonic generation (SHG) microscopy and image analysis

The Nikon A1 RMP confocal microscope equipped with SHG detection was used to characterize collagen fiber qualities in tumor tissues at an excitation wavelength of 870 nm.

Frozen sections (15 μM) were directly observed under the microscope. Random images were collected to determine the qualitative differences in collagen deposition between control and P4HA1, P4HA2, and double knockdown tumor samples. The same laser power and detector gain settings were used when acquiring all images. Two distinct regions of the tumor tissues were analyzed for each mouse within every group.

Analysis of P4HA mRNA expression in ovarian cancer

To further understand the prognostic significance of P4HA in ovarian cancer, transcriptome and clinical data for ovarian serous cystadenocarcinoma patients was downloaded from The Cancer Genome Atlas (http://cancergenome.nih.gov/). A total of 1435 patients were available in this data set. Patients with ovarian cancer were divided into low and high subgroups based on expression of P4HA1 and P4HA2. The low group was defined as median levels of expression, and the high group was defined as > 2 standard deviations from the median. Survival analysis based on P4HA1 and P4HA2 expression was performed using Kaplan-Meier methods and compared by expression groups using log-rank tests.

Statistical analysis

All data was expressed as mean ± SEM (standard error of the mean). Statistical analyses were performed using GraphPad Prism® 6. Differences in mean values between the two groups were analyzed using the two-tailed Student's t-test. *P value* < 0.05 was considered statistically significant.

Data availability statement: The data generated in this study are available upon request from the corresponding author.

Results

Hypoxia upregulates the expression of P4HA1 and P4HA2

Collagens are the major structural component of ECM. While there are 39 different members of the collagen family, they all share common structural features, such as triple helical conformation and post-translational modifications of proline and lysine residues. Hydroxylation of proline residues in pro-collagen is critical for trimerization and secretion. The 4-hydroxyproline modification is ubiquitous in all collagens and confers thermal stability. Three isoenzymes (P4HA1, P4HA2, and P4HA3), collagen prolyl 4-hydroxylases encoded by three distinct genes, have been identified to carry out post-translational modification of collagens. P4HA1 and P4HA2 are more abundant in cells, and therefore, we focused our studies on these two isoenzymes in ovarian cancer cells. We first investigated the role of hypoxia in regulating P4HA expression by determining the transcript levels in A2780 and OVCAR8, human ovarian cancer cell lines. These studies revealed that P4HA1 (**Figure 1A** and **1B**) and P4HA2 (**Figure 1C** and **1D**) mRNA levels were significantly upregulated when cells were exposed to hypoxic conditions compared to normoxia. Additionally, in OVCAR8 cells, when the normoxic environment was restored after the cells were exposed to hypoxia for 24 hours, the expression of P4HA1 and P4HA2 returned to baseline (**Figure 1E**).

P4HA affects collagen secretion

We next sought to evaluate the impact of P4HA on collagen secretion. We used the A2780 cell line model to stably knockdown P4HA gene expression, which was confirmed at the protein levels (**Figure 2A** and **2B**) of a representative type. Genetic knockdown of P4HA resulted in reduced levels of type 1 pro-collagen alpha-1 secretion. Reduced P4HA expression correlated with significantly reduced levels of pro-collagen I alpha 1 secretion in the sh-P4HA1, sh-P4HA2, and sh-P4HA1/2 transfected cells compared to the sh-control cells by 48%, 58%, and 47%, respectively (**Figure 2C**). Interestingly, the reduction of both P4HA 1 and 2 did not produce additive or synergistic effects on collagen Type 1 secretion.

Reduced P4HA inhibits cell migration and proliferation

We then examined the effect of P4HA down regulation on the migration of ovarian cancer cells in a scratch wound assay. Knockdown of either P4HA1, P4HA2, or both showed significant impairment in cell migration and wound closure by approximately 50% compared to control cells (**Figure 3A** and **3B**).

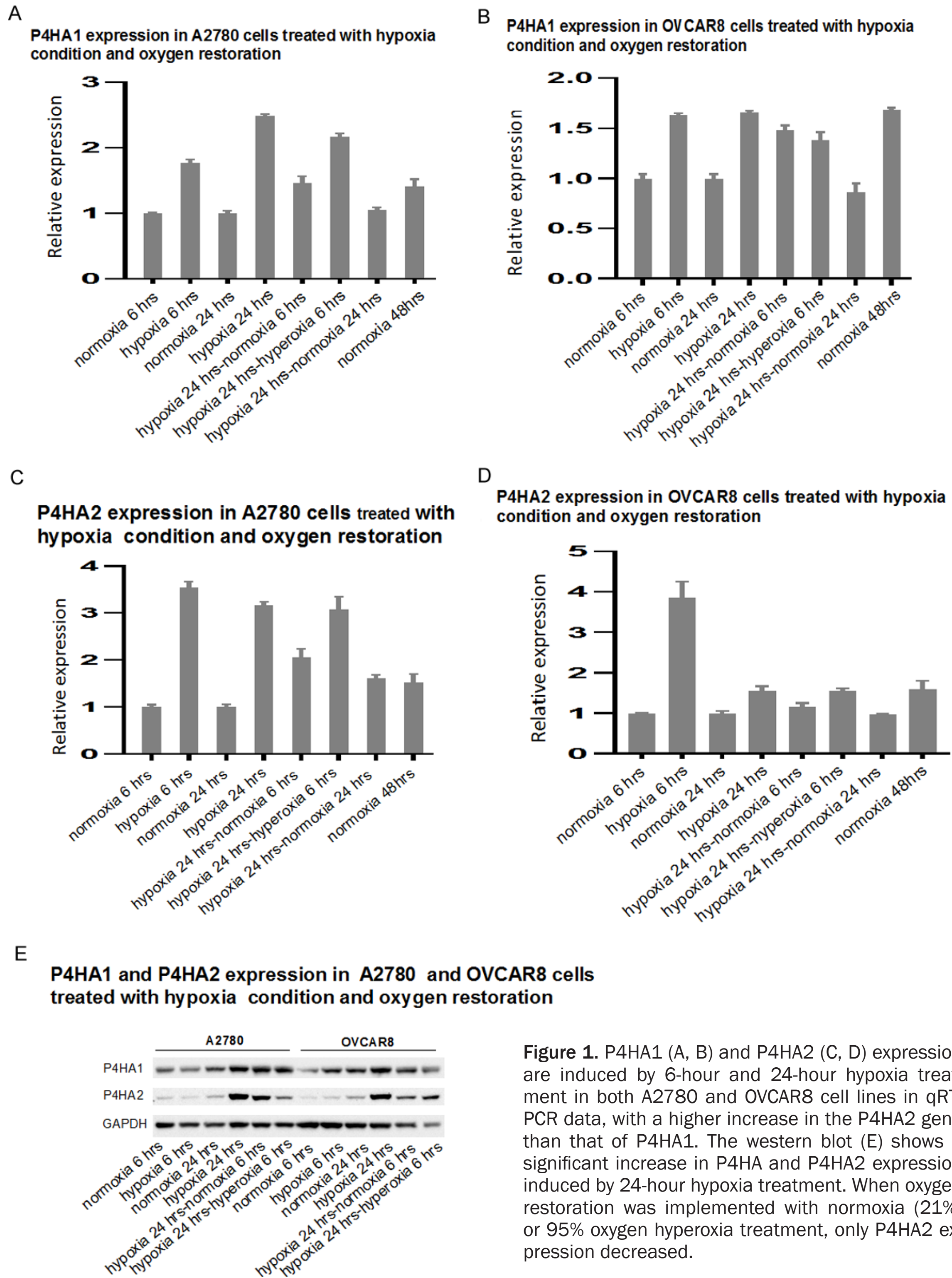

Figure 1. P4HA1 (A, B) and P4HA2 (C, D) expression are induced by 6-hour and 24-hour hypoxia treatment in both A2780 and OVCAR8 cell lines in qRT-PCR data, with a higher increase in the P4HA2 gene than that of P4HA1. The western blot (E) shows a significant increase in P4HA and P4HA2 expression induced by 24-hour hypoxia treatment. When oxygen restoration was implemented with normoxia (21%) or 95% oxygen hyperoxia treatment, only P4HA2 expression decreased.

We next examined the effect of P4HA on ovarian cancer cell proliferation in real-time. Prolyl hydroxylase knockdown was associated with decreased proliferation, as measured by the change in electrical impedance depicted as the 'cell index' (**Figure 3C**). Similarly, the simultaneous knockdown of both P4HA1 and P4HA2 did not result in a further reduction in cellular pro-

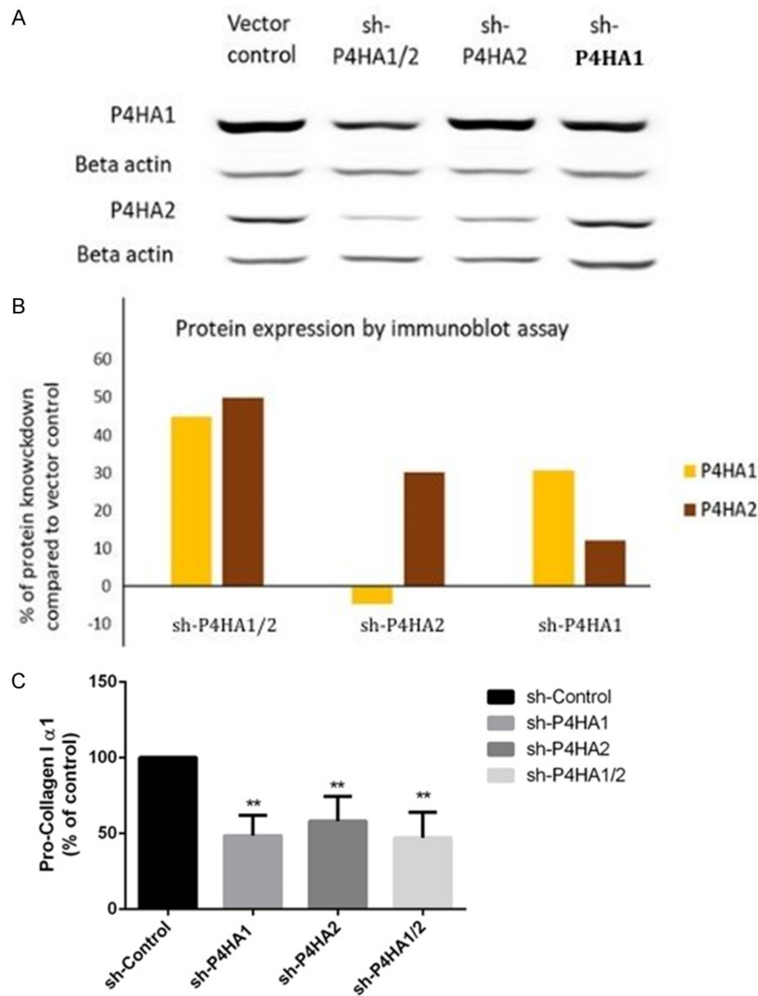

Figure 2. A2780 cells were stably transfected with shRNA specific for P4HA1 and P4HA2. A. Western blot analysis of P4HA expression in sh-P4HA1 and sh-P4HA2 transfected cells. B. Bar chart representing the percentage of P4HA1 or P4HA2 knockdown in these transfected cells. C. ELISA analysis shows decreased collagen concentrations in the ECM of all transfected sh-P4HA cells. Bar and error bar refer to the mean and SEM of three experiments (**$P \leq 0.01$).

liferation (viability) compared to the knockdown of either gene alone.

Knockdown of P4HA reduces peritoneal seeding and growth of ovarian cancer cells in vivo

To determine the biological significance of our *in vitro* results, we examined the impact of P4HA in an ovarian cancer xenograft mouse model. We initially determined the early events of tumor cell seeding in the peritoneum. Control and P4HA1/2 knockdown tumor cells were injected intraperitoneally (IP) (n=7 mice per group). One week after tumor cell injection, mice were sacrificed, and the number of peritoneal tumor nodules was counted. **Figure 4A** illustrates that double P4HA1/2 knockdown significantly inhibited early peritoneal seeding with a mean of four nodules per mouse (range 0-7, 58% reduction) compared to the sh-control tumor cells (mean of ten tumor nodules, range 5-19). These data strongly suggest that prolyl hydroxylases are important in ovarian cancer seeding and spreading within the peritoneal cavity.

Next, we researched the effect of P4HA on tumor growth (n=12 mice per group). Four mice in the sh-P4HA2 group and one mouse in the sh-control group died before week five and were excluded from the analysis. To allow for tumor growth, we sacrificed all mice by week 5. The number of tumor nodules per mouse in the sh-P4HA1, sh-P4HA2, and sh-P4HA1/2 knockdown mice were significantly lower when compared to the mice with sh-control vector control (**Figure 4B**). The mean weight of the tumors per mouse in the sh-control group was 6.4 grams. P4HA1 knockdown had a moderate reduction in mean tumor weight, which was statistically significant. P4HA2 knockdown showed better inhibition of tumor growth and had a 55% reduction in mean weight per mouse (2.9 grams, P=0.0085). Further inhibition of tumor growth was observed when both P4HA1 and P4HA2 were knocked down with a mean weight of only 1.9 grams, a 70% reduction in tumor burden when compared to the control group of animals (**Figure 4C** and **4D**).

P4HA changes tumor collagen structure

By using the confocal microscopy we investigated the effect of the knockdown of P4HA on extracellular collagen in mouse tumor sections by Second Harmonic Generation. Represen-

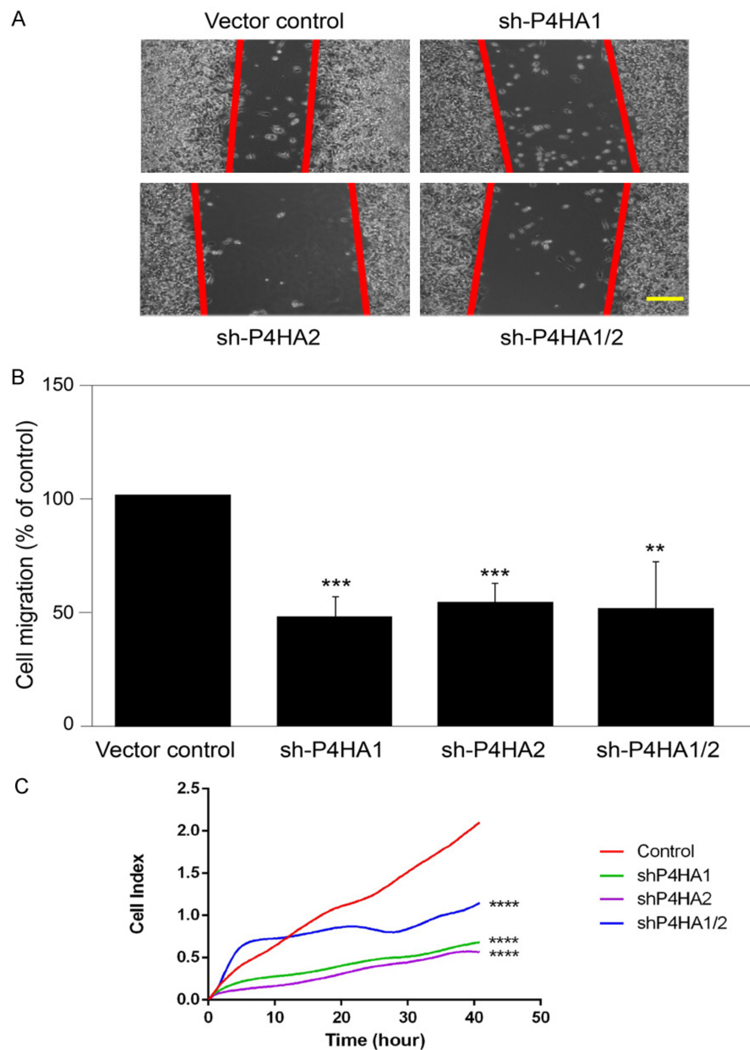

Figure 3. P4HA modulation affects migration of A2780 ovarian cancer cells. A. Representative wound healing assay in sh-P4HA1, sh-P4HA2, and sh-P4HA1/2 cells compared to relative sh-control cells at 24 hr. The scale bar (yellow) shown in A represents 50 um. B. Bar chart represents the percentage of wound residual distance relative to time 0 hr. Bar and error bar refer to mean and SEM of three experiments. C. Electrical impedance measurements show decreased cell proliferation of transfected cells (*P < 0.05; **P ≤ 0.01; ***P ≤ 0.001).

tative images from two tumor samples are shown in (**Figure 4E**). Tumor sections from vector control samples exhibited relatively higher levels of wavy patterns of collagen fibers. Individual knockdown of P4HA1 or P4HA2 showed decreased amounts of wavy collagen, while the more dramatic linear fibers were seen in the double knockdown samples. Some of the areas of the tumors showed thicker, linearly oriented collagen fibers with limited amounts of mesh-like structures. Previous studies have shown ovarian tumors have a highly wavy pattern (SHG) of collagen deposition [24]. In comparison, normal ovaries were found to have less crosslinked and linear organization of collagen. In the present studies, P4HA knockdown resulted in collagen fibers that resembled the pattern seen in normal ovaries.

P4HA expression correlates with human ovarian cancer prognosis

To determine the clinical relevance of our data, we first analyzed the data set available on ovarian serous cystadenocarcinoma (Nature 2011, array data). In this analysis, we determined the correlation between P4HA1 downregulation and survival. Of 316 patients, 6% showed lower expression of P4HA1. Lower expression correlated with improved median overall survival compared to the median expression levels of P4HA1 (68.9 vs. 43.9 months, P= 0.0166) (**Figure 5A**). Conversely, patients with tumors overexpressing both P4HA1 and P4HA2 (> 2 SD) had significantly decreased overall survival compared to median levels of both P4HA1 and P4HA2 (24.2 vs. 44.5 months, respectively, P=0.00065, data not shown).

P4HA2 expression was then analyzed using the TCGA provisional data set on ovarian serous cystadenocarcinoma patients. A total of 303 tumor RNA seq data are available in this data set. Nine patients in this data set had P4HA2 RNA levels > 2 SD from the median levels of expression. Overall survival of these patients with higher P4HA2 expression was 16.2 months compared to 44.48 months survival of patients with median expression levels (P=0.00619, **Figure 5B**). These results suggest that overexpression of prolyl hydroxylases, and conse-

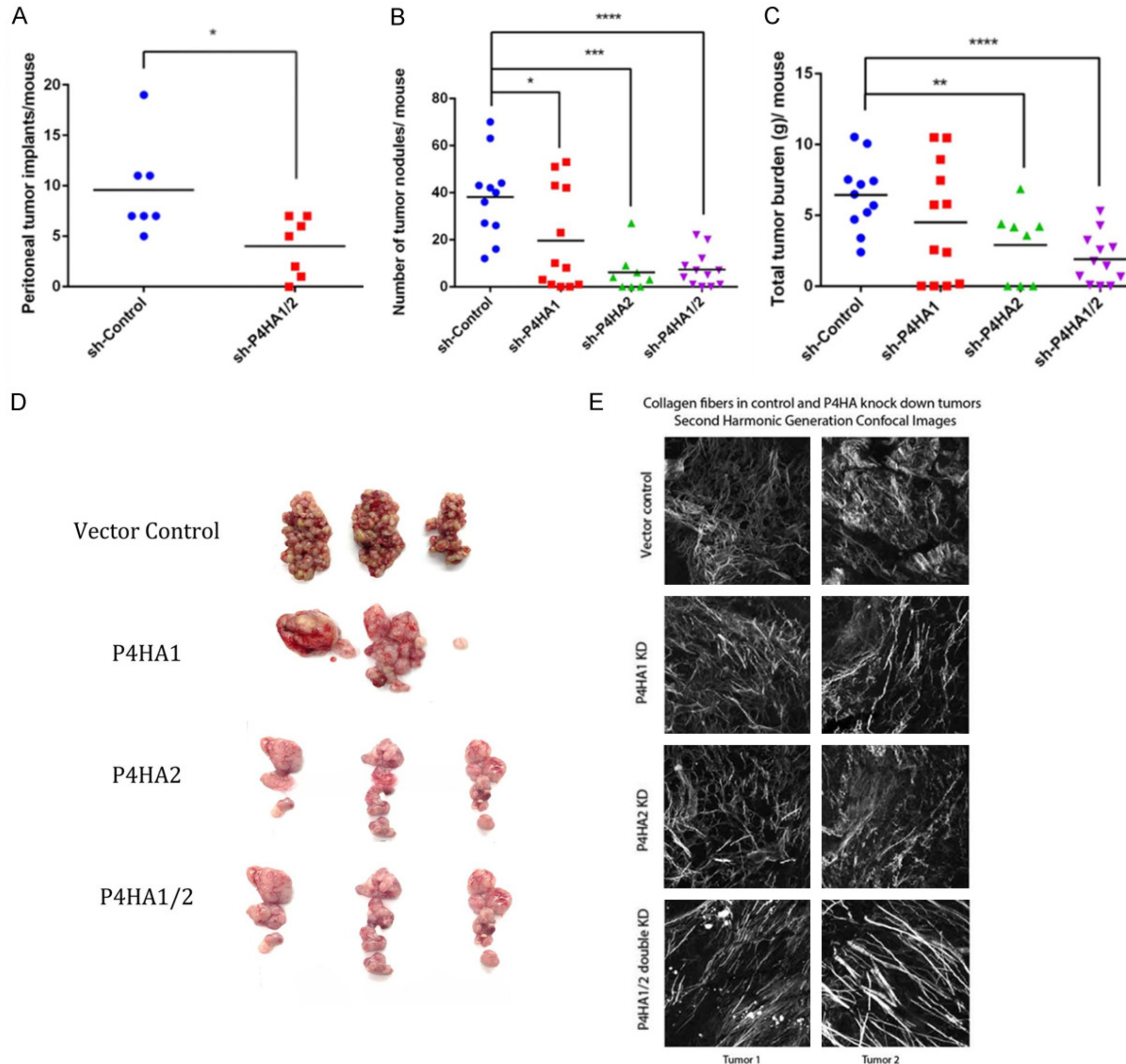

Figure 4. (A) Decreased expression of prolyl hydroxylases reduces peritoneal seeding of ovarian cancer cells. (B) Decreased expression of prolyl hydroxylases reduces the number of tumor nodules, and (C) means total weight of the tumor nodules. (D) Tumor burden study. (E) Knockdown of prolyl hydroxylases affects the content and quality of collagen fibers (*P ≤ 0.05; **P ≤ 0.01; **P ≤ 0.001; ****P≤ 0.0001).

quently the collagen deposition in the TME, negatively correlate with the survival of ovarian cancer patients.

Discussion

P4HA impacts multiple aspects of tumor biology, including cell proliferation, migration, and invasion. Our studies show that P4HA knockdown in ovarian cancer cells reduced procollagen secretion, thereby altering and remodeling the ECM components produced by cancer cells. We demonstrated the impact of P4HA on peritoneal seeding, tumor growth, and metastasis in ovarian cancer. In addition, higher levels of P4HA expression were associated with poor clinical outcomes in patients with ovarian cancer in TCGA. These studies support the idea that post-translational modification of collagen is crucial to the spread and growth of ovarian cancer cells.

Previous studies have focused on the late stages of collagen maturation and crosslinking through the lysyl oxidases (LOX) family and its role in ovarian cancer. Although several studies have demonstrated that P4HAs are essential for invasion and metastasis in other cancers, this is the first study to evaluate the role of P4HA1 and P4HA2 in the modulation of the

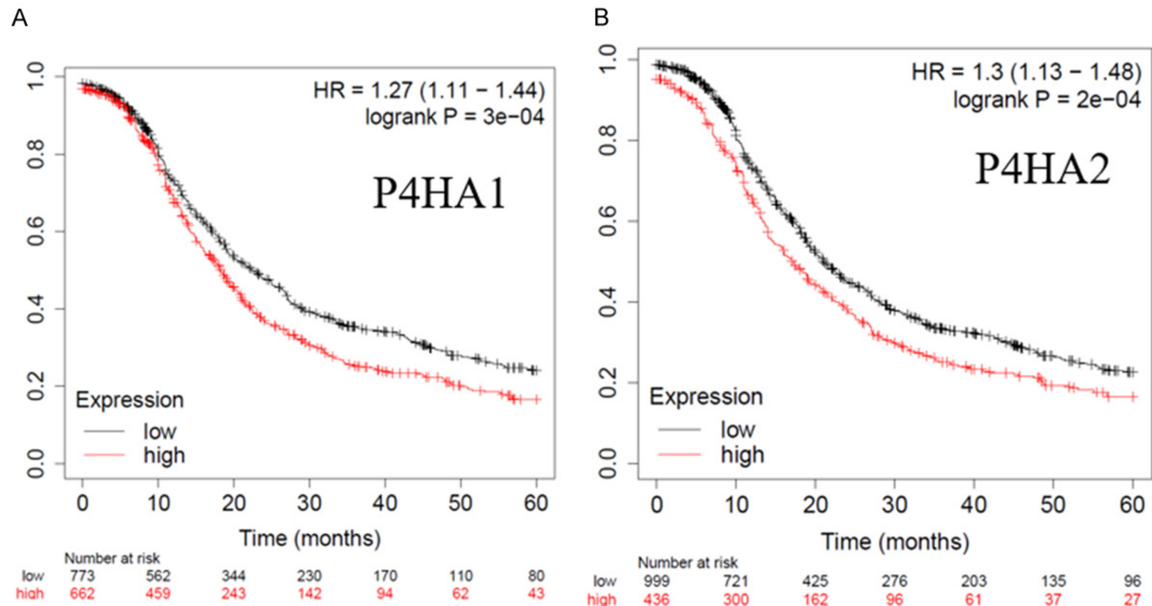

Figure 5. Kaplan-Meier overall survival analysis shows that ovarian patients with higher P4HA1 (A) and P4HA2 (B) expression displayed significantly poorer overall survival than those with lower P4HA1 and P4HA2 expression (Total patients: 1435).

tumor microenvironment and metastasis in ovarian cancer [2, 25, 26]. Collagen prolyl hydroxylases may play a significant role in facilitating the ovarian cancer progression and have a prognostic significance.

Our data suggest that hypoxia affects collagen production. HIF-1alpha is activated in the hypoxic environment of cancer cells and promotes metastasis by regulating multiple genes involved in angiogenesis, glucose metabolism, and the tumor microenvironment [7]. Several studies have shown that HIF-1alpha can regulate collagen biosynthesis at multiple stages of production. HIF-1alpha regulates the expression of P4HA1 and PH4A2 in both cancer cells and non-cancer cell lines [13, 16, 27]. Collagen deposition from breast cancer cells and fibroblasts *in vitro* is reduced by decreased expression of HIF-1, P4HA1, or P4HA2. Furthermore, tumors formed by human breast cancer cells expressing lower levels of P4HA1 or P4HA2 induced reduced fibrosis and stiffness when injected into mice [17]. Thus, HIF-1 is needed for both the early and late stages of collagen fiber formation and is prognostic for survival. In patients with ovarian cancer, HIF-1 protein and p53 overexpression were significantly associated with decreased overall survival (P=0.0001) [28]. The promoter region of P4HA1 contains a conserved HIF-1 response element (HRE), and HIF-1 has been shown to regulate P4HA1 transcription by binding to the HRE in the P4HA1 promoter [13]. Interestingly, recent studies have revealed a positive feed-forward loop between HIF-1 and P4HA1. It is well-known that HIF-1alpha is targeted for proteasomal degradation through hydroxylation of prolyl residues by PHD enzymes. The cofactors for PHDs include α-ketoglutarate, ascorbic acid, Fe++, and oxygen. P4HA1 exhibits a 3-fold higher affinity for α-ketoglutarate and a 6-fold higher affinity for O_2 when compared to PHD enzymes. The higher level of P4HA1 sequesters a significant amount of the substrates needed by PHD enzymes, thereby reducing the proteasomal degradation of HIF-1α [29, 30]. Under hypoxia, a complex network of interconnected pathways reinforces the extracellular matrix. Initially, hypoxia suppresses the TCA cycle, reducing the availability of α-ketoglutarate. Consequently, lower levels of essential substrates, specifically α-ketoglutarate and oxygen, inhibit PHD enzymes, thereby stabilizing HIF-1alpha by preventing proteasomal degradation. This stabilized HIF-1alpha then triggers the activation of P4HA1 transcription. As P4HA1 levels increase, they sequester available α-ketoglutarate and O_2, further enhancing HIF-1 stabilization. Once collagen is secreted beyond the cancer cells,

HIF-induced expression of Lysyl Oxidases comes into play. This process facilitates the cross-linking and remodeling of collagen fibers, ultimately promoting cancer metastasis and enhancing resistance to chemotherapy [31, 32].

Our findings align with other studies showing a critical role for P4HA in collagen secretion, cancer cell migration, and metastasis. P4HA1 or P4HA2 knockdown significantly inhibited lung and lymph node metastasis of breast cancer cells in mice by reducing collagen formation [16, 33]. P4HA1 knockdown also considerably decreased aggressive prostate cancer cell proliferation, motility, and invasion in both *in vitro* and *in vivo* murine xenograft studies [18]. Duan et al. found that miR-122 targets P4HA1 transcripts in SKOV3 cells and that [34] overexpression of miR-122 decreased tumor growth and metastasis potentials. These studies indirectly implicate P4HA1 in ovarian cancer growth, and our current studies provide direct evidence to show that P4HA is vital in enhancing ovarian cancer growth and metastasis.

Collagen content affects the biophysical properties of the ECM and can promote cancer progression [7, 35]. Collagen content and quality changes can impact the tumor stroma's stiffness, density, and architecture, influencing tumor cell behavior, including their ability to invade surrounding tissues and metastasize to distant sites. Intracellular modifications of procollagen fibers are essential for the production of mature collagen fibrils with the appropriate mechanical properties, enabling them to fulfill their structural roles in the body. The alignment of collagen fibers has been shown to play an important role in directing cancer cell migration *in vivo* [33, 36]. Future studies will address the quality and quantity of cross-linked collagen fibers in the ovarian tumor stroma when prolyl hydroxylases are knocked down.

Our analysis from the TCGA dataset suggests that overexpression of prolyl hydroxylases, and consequently collagen synthesis, negatively correlates with the overall survival of ovarian cancer patients [16, 17, 33]. A meta-analysis classifying genes that are upregulated across many different cancer types identified with P4HA1. P4HA1 and P4HA2 expression have been shown to be prognostic of survival for patients with breast cancer and pancreatic ductal adenocarcinoma [37]. Our findings thus strengthen the hypothesis that P4HA activity in ECM remodeling plays a critical prognostic role in ovarian cancer metastasis and survival.

Our study provides compelling evidence that, under hypoxic conditions, both P4HA1 and P4HA2 expression is significantly upregulated, which leads to notable changes in the secretion of collagen into the extracellular matrix (ECM) and a subsequent alteration in the structure of collagen fibers. Significantly, these alterations contribute to an increased metastatic potential. As P4HA levels correlate with the overall survival of ovarian cancer patients, our studies highlight collagen prolyl hydroxylases as a promising therapeutic target and perhaps a biomarker for ovarian cancer progression.

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Disclosure of conflict of interest

None.

Address correspondence to: Mihae Song, Division of Gynecologic Oncology, Department of Surgery, City of Hope National Medical Center, Duarte, CA 91010, USA. E-mail: misong@coh.org

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