Hypoxia promotes HO-8910PM ovarian cancer cell invasion via Snail-mediated MT1-MMP upregulation

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Abstract

The molecular mechanisms of ovarian cancer cell invasion under hypoxia remain unclear. Here we employed a 3D collagen model and chick chorioallantoic membrane (CAM) invasion assay to explore the influence of hypoxia on ovarian cancer cell invasion. Hypoxia (both 1% O₂ and CoCl₂ 150 and 250 μM) induced HO-8910PM ovarian cancer cell invasion in 3D collagen and collagenolysis determined by hydroxyproline. Pretreatment with a hypoxia inducible factor-1α inhibitor, YC-1, or MMP inhibitor, GM6001, significantly inhibited 3D collagen invasion and degradation and cell proliferation. Hypoxia stimulated both mRNA and protein expressions of membrane-type 1 matrix metalloproteinase (MT1-MMP) and promoted MT1-MMP translocation to the cell surface in an YC-1 sensitive manner. MT1-siRNA transfection inhibited hypoxia-induced invasion, proliferation, and collagen degradation of cells in 3D collagen. Hypoxia stimulated Snail mRNA and protein expression as well as translocation to nucleus in an YC-1 sensitive manner. Overexpression of Snail with a recombinant plasmid in HO-8910PM cells resulted in an enhanced invasion in 3D collagen. Transfection with Snail-specific siRNA significantly decreased MT1-MMP expression and 3D collagen invasion. Hypoxia-treated cells significantly broke the upper CAM surface of 11-day-old chick embryos and infiltrated interstitial tissue, completely blocked in the presence of YC-1 or GM6001, or after MT1-MMP siRNA or Snail siRNA transfection. Together, these data suggest that hypoxia promotes HO-8910PM ovarian cancer cell traffic through 3D matrix via Snail-mediated MT1-MMP upregulation, a possible molecular mechanism of ovarian cancer cell invasion under hypoxia.

Keywords: Hypoxia, ovarian cancer, Snail, three-dimensional extracellular matrix, membrane-type 1 matrix metalloproteinase, invasion

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Introduction

Ovarian cancer is a common cancer of the female reproductive organs, with the highest mortality rate of gynecological cancers.¹ The case-specific mortality rate of diagnostic ovarian cancer within one year is approximately 70%.² In patients with ovarian cancer undergoing laparotomy, only 30% of tumors were confined to the ovary, while the majority metastasized to the uterus, bilateral annex, peritoneum, and pelvic organs.

Hypoxia is one of the basic characteristics of solid tumors^{3,4} and stimulates tumor cell proliferation, invasion, and metastasis.^{5–8} Extracellular matrix (ECM) is an inevitable obstacle for cancer cell invasion and metastasis, while some invasive cancer cells can destroy ECM and invade stromal tissue.^{9,10} Membrane-type 1 matrix metalloproteinase (MT1-MMP), an effective local proteolytic enzyme, plays an important role in tumor invasion.^{11–17}

Recently, we found Snail controls MT1-MMP expression and invasion of bone marrow mesenchymal stem cells when triggering with platelet-derived growth factor-BB (PDGF-BB).¹⁸ Although Snail has been extensively researched as an inhibitor of E-cadherin expression, initiating the epithelial-mesenchymal transition during tumor progression and embryonic development,^{19–21} whether Snail involves hypoxic ovarian cancer cell traversing through 3D collagen gels remains undefined.

In the present study, we employed a 3D collagen gel model and chick embryo to mimic the *in vivo* cellular environment and explore the influence of hypoxia on ovarian cancer cell invasion and the molecular mechanisms. Herein, we demonstrate that hypoxia induces Snail expression, thereby upregulating MT1-MMP expression and activity, eventually resulting in HO-8910PM ovarian cancer cell traffic through 3D matrix *in vitro* and *in vivo*.

Materials and methods

Cell culture and materials

The highly metastatic human ovarian cancer HO-8910PM cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China).²²⁻²⁵ Cells were cultured in Dulbecco's modified Eagle's medium (Gibco, NY, USA) supplemented with 10% fetal bovine serum (Gibco), in a humidified atmosphere containing 5% CO₂ at 37°C. Hypoxic conditions were created using a hypoxia chamber contained 1% O₂, 5% CO₂, and balanced with N₂ or using CoCl₂ to produce hypoxic conditions. CoCl₂, YC-1, DiI, DAPI, and Alexa Fluor 594-labeled phalloidin were purchased from Sigma-Aldrich (St Louis, MI, USA). GM6001 was from Millipore Corporation (Billerica, MA, USA). Sulfo-NHS-SS-Biotin and Streptavidin-Sepharose beads were from Thermo Fisher Scientific (Rockford, IL, USA). Fluoresbrite carboxylate microspheres were from Polysciences (Warrington, PA, USA). ECL was obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Primers, TRIzol, Superscript First-Strand Synthesis System, LipofectamineTM RNAiMAX transfection reagents, and Alexa Fluor-labeled secondary antibodies were from Invitrogen (CA, USA). All other reagents were from common commercial sources.

3D type I collagen invasion and degradation

The invasion assay was performed as described previously.²⁶ Briefly, HO-8910PM cells (2×10^5) were mixed with 100 μ L of collagen (2.2 mg/mL) extracted from rat tail and gelled in 96-well culture plates at 37°C. After gelling, the cell-collagen mixtures were further embedded in 500 μ L of collagen in 24-well culture plates. Cultures were treated with normoxia (5% CO₂ balanced with atmosphere) or hypoxia (1% O₂ and 5% CO₂ balanced with N₂ or chemical hypoxia with CoCl₂ 150 or 250 μ M) for 24 h. Where indicated, cultures were preincubated with YC-1 or GM6001 for 1 h prior to hypoxia treatment. On the following day, all the cultures were placed under normoxic condition. Cell invasion was observed by phase-contrast microscopy and invading cell numbers were counted in five randomly selected fields (mean $\pm$ SD). Images were captured and invasion depths were measured in five randomly selected fields in a representative experiment of three at least (mean $\pm$ SD). For 3D collagen degradation assays, the culture medium was collected and collagen degradation product, hydroxyproline, was quantified as described previously.²⁷ Results are presented as the mean $\pm$ SEM of three independent experiments.

Growth and proliferation in 3D type I collagen

To observe HO-8910PM cells growth and proliferation in 3D ECM, 2×10^5 HO-8910PM cells were mixed with 100 μ L of collagen in 96-well plates. After gelling, 50 μ L growth medium was added, supplemented with or without CoCl₂ 150 or 250 μ M. Where indicated, cultures were preincubated with YC-1 or GM6001 for 1 h prior to hypoxia treatment. Over a three-day culture period (first day under either normoxia or hypoxia, the following two days under normoxia),

images were captured and cell length was measured in three randomly selected fields (mean $\pm$ SD). Cell numbers were determined after the collagen gel was dissolved by collagenase II (mean $\pm$ SEM, $n = 3$).

RT-PCR analysis

Cells were cultured under normoxia or hypoxia (1% O₂) for 2–4 h and total RNA was extracted using TRIzol reagent and reverse-transcribed into cDNA using Superscript First-Strand Synthesis System. cDNA was amplified using PCR with specific primers, MT1-MMP (sense 5'-aaccaagtgatggatgatacc-3'; antisense 5'-atccagaagagagcagcatca-3', 272-bp product), Snail (sense 5'-ccaatcggaagcctaactacag-3'; antisense 5'-gacagagtcccagatgagcatt-3', 155-bp product), glyceraldehyde 3-phosphate dehydrogenase (sense 5'-AAGAAGGTGGTGAAGCAGGC-3'; antisense 5'-TCCA CCACCCAGTTGCTGTA-3', 287-bp product) as an internal reference. RT-PCR products were separated on 1.5% (w/v) agarose gels and photographed by an AlphaEase Digital Imaging System (Alpha Innotech, San Leandro, CA). Bands were analyzed using Quantity One software (Bio-rad, CA, USA; mean $\pm$ SEM, $n = 3$).

SiRNA transfection

Stealth[®] RNAi directed against Homo MT1-MMP (NM_004995.3), MT1si (5'-AACAGGCAAAGC TGATGCAGA-3' nt 228–248), or against Homo Snail1 (NM_005985.3), snsi (5'-aatgctcatctgggactctgt-3'; nt 171–191), was transfected into cells with LipofectamineTM RNAiMAX (Invitrogen), using Duplex StealthTM 7RNAi as negative control. Knockdown efficiency of MT1-MMP or Snail was determined by RT-PCR 24 h after transfection.

Western blotting analysis

HO-8910PM cells 70% confluent were treated with either normoxia or hypoxia (1% O₂ or CoCl₂) for 24 h or pretreated with YC-1 prior to hypoxia treatment. After 24 h treatment, protein was extracted and subjected to 10% SDS-PAGE and transferred onto nitrocellulose membranes. To detect multiple antigens on the same membrane, antibodies binding to the membrane were stripped off using stripping buffer and re-incubated with additional antibodies. All antibodies used as following: mouse anti-HIF-1 α antibody (1:1000) from BD Biosciences (NJ, USA), rabbit anti-MT1-MMP hinge region antibody (1:1000) from Millipore Corporation (Billerica, MA, USA), rabbit anti-Snail antibody (1:200) and mouse anti-actin antibody (1:20000) from Santa Cruz Biotechnology (CA, USA). Protein bands were quantified using Quantity One software (Bio-Rad) and presented as mean $\pm$ SEM of three independent experiments.

Cell surface biotinylation

Cell monolayers were washed twice with ice-cold PBS and incubated with Sulfo-NHS-SS-Biotin (0.5 mg/mL) on ice for 30 min. Cells were then incubated with lysis buffer after three washes with cold PBS. Cell lysates were collected and incubated with Streptavidin-Sepharose beads for 1 h at 4°C. The captured biotinylated proteins were then

analyzed with SDS-PAGE followed by Western blotting with anti-MT1-MMP antibodies. An aliquot of total cell lysates is used to examine β -actin with Western blotting as loading control.

Fluorescent immunocytochemistry

Fluorescent immunocytochemistry assays were performed as described previously. Briefly, cells were fixed in 4% paraformaldehyde for 10 min followed by 0.25% Triton X-100 treatment for 5 min. After blockade with 3% goat serum, the cells were incubated with rabbit anti-MT1-MMP antibody (1:25) or anti-Snail antibody (1:50) for 2 h and followed by incubation with Alexa Fluor 488-labeled anti-rabbit secondary antibody (1:1000) for 1 h. Where indicated, cell-surface MT1-MMP was stained with anti-MT1-MMP antibody for 2 h at 4°C and cells were then permeabilized with 0.25% Triton X-100 and incubated with Alexa Fluor 488-labeled secondary antibody. The cells were counterstained with 4',6-diamidino-2-phenylindole (1:200) for nucleus, DiI (1:100) for membrane, and Alexa Fluor 594-labeled phalloidin (1:200) for cytoskeleton, and fluorescence was observed by confocal laser microscopy.

CAM invasion

CAM invasion assay was performed as described previously.^{26,28} Briefly, cells were labeled with green Fluoresbrite carboxylate microspheres and seeded atop CAM of 11-day-old chick embryos and incubated for two days. Frozen sections of samples were incubated with anti-type IV collagen and Alexa Fluor 594-labeled secondary antibody (Invitrogen). Fluorescent images were captured by fluorescence microscopy. Invasion is quantified as the number of HO-8910PM cells that cross the CAM surface and infiltrate the underlying stromal tissues (mean $\pm$ SEM, $n=3$).

Results

Hypoxia induces HO-8910PM ovarian cancer cell invasion in 3D collagen

To examine human ovarian cancer cell invasive ability, type I collagen, the major extracellular protein found in mammals, was extracted from rat tails and used to generate 3D structures, mimicking the structural characteristics of the interstitial matrix *in vivo*.^{29,30} HO-8910PM ovarian cancer cells were enmeshed within 3D type I collagen gels and invasive activities were observed under normoxia (20% O₂) and hypoxia (1% O₂). After a two-day culture, although HO-8910PM cells showed invasive activity under normoxic conditions, more significant invasion was seen when cells were treated with 1% O₂, with 2.6- and 2.3-fold higher invasion depth and invasive cell number, respectively, compared with normoxic cultures (Figure 1(a) and (b)). YC-1 can inhibit the expression of HIF-1 α and promotes HIF-1 α transfer from nucleus to cytoplasm.³¹ In HO-8910PM cells, YC-1 not only decreased the expression of MT1-MMP, but also inhibited tumor cell invasion and proliferation. Furthermore, the observed increased invasion of cells

under hypoxia was markedly blocked by pretreatment with YC-1 (Figure 1(a) and (b)).

To test whether MMPs play a role in HO-8910PM cell invasion in 3D collagen, GM6001, an inhibitor of MMPs, was employed. Results showed that HO-8910PM cell invasion in 3D collagen, both in terms of invasion depth and invasive cell number, was completely blocked by pretreatment of cells with GM6001 (Figure 1(a) and (b)). To further examine the effect of hypoxia on HO-8910PM cell invasion, CoCl₂ (150 and 250 μ M) was used to produce hypoxic conditions. Significantly increased cell invasion in 3D collagen, both in terms of invasion depth and invasive cell number, was observed in HO-8910PM cells treated with CoCl₂ at both concentrations compared with controls (Figure 1(a) and (b)). In contrast, CoCl₂-induced cell invasion was significantly inhibited in the presence of YC-1 (Figure 1(a) and (b)). Next, we evaluated collagen degradation by detecting a typical degradation product, soluble hydroxyproline, in the collected cell culture medium. Results showed that amount of hydroxyproline significantly increased in HO-8910PM cells after treatment with both 1% O₂ and CoCl₂, whereas pretreatment with YC-1 or GM6001 significantly inhibited collagen degradation (Figure 1(e)), indicating that HO-8910PM cells invade and degrade 3D type I collagen in a HIF-1 α - and MMP-dependent manner.

Hypoxia promotes HO-8910PM cell growth and proliferation in 3D collagen

Although hypoxia upregulates 3D collagen degradation and invasion abilities of HO-8910PM cells, whether HO-8910PM cell proliferation in 3D collagen is influenced by hypoxia has not been explored. To examine proliferation, HO-8910PM cells were embedded in 3D collagen gels in the absence or presence of hypoxia or CoCl₂ (150 and 250 μ M). When treated with hypoxia (1% O₂ or CoCl₂), HO-8910PM cells exhibited a marked increase in cell length in 3D collagen compared with the cells in normoxic conditions (Figure 2(a) and (b)). When treated with YC-1, however, HO-8910PM cells did not exhibit the growth in length (Figure 2(a) and (b)). Consistent with these observations, the proliferative response of HO-8910PM cells increased under 1% O₂ hypoxic conditions or in the presence of CoCl₂ in an YC-1 sensitive manner (Figure 2(a) and (c)). These increases in cell length and proliferation induced by hypoxia were inhibited almost completely by pretreatment with GM6001, indicating that HO-8910PM cell growth and proliferation in 3D type I collagen occurs in a HIF-1 α - and MMP-dependent manner (Figure 2(a) to (c)).

Hypoxia upregulates MT1-MMP expression and localization in HO-8910PM cells

Although 3D invasion assay demonstrated that hypoxia could promote invasion of HO-8910PM cells, the molecular factors involved in the regulation of cell invasion under hypoxia (1% O₂ or CoCl₂) are unknown. RT-PCR, western blot, and fluorescence immunocytochemistry were performed to detect the expression of MT1-MMP in HO-8910PM cells under normoxia or hypoxia. As shown in Figure 3(a) and (b), weak expression of MT1-MMP was

Figure 1 Hypoxia induces HO-8910PM ovarian carcinoma cell invasion in 3D type I collagen. (a, b) HO-8910PM ovarian cancer cells enmeshed within 3D collagen gels were cultured under normoxia (NOR, 20% O₂) or hypoxia (HY, 1% O₂) for 24 h and all the cultures were cultured under normoxia for another 24 h. A total of 100 μM YC-1 and 25 μM GM6001 was used to block HIF-1α and MMP, respectively. Invasion was monitored by phase-contrast microscopy, with white dashed lines demarcating the boundaries between the inner and outer collagen gels, blue arrows indicating the HO-8910PM cells invading into the outer collagen gels, and red arrow heads marking the HO-8910PM cells staying in the originally central collagen gels. Invasion depth was measured (mean ± SD, n = 5) and invading cell numbers per field were counted (mean ± SD, n = 5). * *P* < 0.01. (c, d) 3D collagen invasion of HO-8910PM ovarian carcinoma cells treated with CoCl₂ (150 or 250 μM) or treated with YC-1 (100 μM) prior to the addition of CoCl₂ was monitored by phase-contrast microscopy. Invasion depth and invading cell numbers (mean ± SD, n = 5). * *P* < 0.01. (e) 3D collagen cultural medium was collected and hydroxyproline in the medium was quantified (mean ± SEM, n = 3). * *P* < 0.01. Scale bars: 100 μm. (A color version of this figure is available in the online journal.)

observed in HO-8910PM cells cultured under normoxic condition by RT-PCR and western blot. After treatment with both 1% O₂ and CoCl₂, expression of MT1-MMP at both the mRNA and protein level was significantly

upregulated in HO-8910PM cells, and this upregulation was blocked in the presence of YC-1 (Figure 3(a) to (c)). Consistent with RT-PCR and western blot results, fluorescence immunocytochemistry results showed that in

Figure 2 Hypoxia promotes HO-8910PM cell growth and proliferation in 3D collagen. (a, b) HO-8910PM cell growth in 3D collagen gels under normoxia (NOR) or hypoxia condition (HY 1% O₂, or CoCl₂ 150 or 250 μM) with or without inhibitors (YC-1 100 μM, GM6001 25 μM) for three days (first day under either normoxia or hypoxia, the following two days under normoxia), images were captured and cell length was measured (mean ± SD, n = 3). Red double-headed arrows demarcate cell length in 3D collagen gels. Scale bars: 100 μm. (c) Fold of increase in cell number under normoxic or hypoxic conditions (mean ± SEM, n = 3). *P < 0.01. (A color version of this figure is available in the online journal.)

comparison with the weak staining of MT1-MMP in normoxic cells, after treatment with hypoxia, MT1-MMP was clearly observed prominently in plasma. In contrast, no MT1-MMP staining could be seen in HO-8910PM cells pretreated with YC-1, indicating that MT1-MMP protein expression is upregulated by hypoxia (Figure 3(d) and (e)).

To further examine the functional MT1-MMP, cell surface biotinylation followed by western blot was performed to detect MT1-MMP expression on the cell surface. As shown in Figure 3(f) and (g), we detected significant increases in a 55 kDa band, active MT1-MMP, on the surface of the cells treated with hypoxia, whereas only a very weak 55 kDa band of active MT1-MMP on the cell surface was observed in the presence of YC-1. Consistent with cell surface biotinylation results, immunofluorescence staining showed that compared with background MT1-MMP on

the cell surface, the expression of surface MT1-MMP increased after 24 h hypoxic treatment in an YC-1 sensitive manner (Figure 3(h) and (i)).

MT1-siRNA inhibits hypoxia-induced invasion, proliferation, and collagen degradation of HO-8910PM cells in 3D collagen

To directly assess the role of MT1-MMP under hypoxic conditions in HO-8910PM cells in 3D collagen, HO-8910PM cells were transfected with homo MT1-MMP-specific siRNA (MT1si) or scrambled siRNA (scrsi) as negative control. MT1-MMP silencing was confirmed by RT-PCR and western blot (Figure 4(a) and (b)). To examine the effects of MT1-MMP depletion on invasive abilities of HO-8910PM cells, siRNA-transfected cells were cultured in

Figure 3 Hypoxia upregulates MT1-MMP expression and cell surface localization in HO-8910PM cells. (a) RT-PCR analysis of MT1 mRNA expression in HO-8910PM cultured under normoxic (NOR) or hypoxic conditions (HY 1% O₂ or CoCl₂ 150 or 250 μM) or in the presence of YC-1 (100 μM). (b, c) Western blotting analyses of HIF-1α and total MT1 protein expression in HO-8910PM treated with or without hypoxia (1% O₂ or CoCl₂) or in the presence of YC-1. Protein bands were measured by densitometry and normalized to actin. (d, e) Fluorescence immunocytochemistry analysis of MT1-MMP in HO-8910PM cells cultured under normoxic or hypoxic conditions or in the presence of YC-1 (100 μM), monitored by confocal laser microscopy. Scale bars: 50 μm. (f, g) MT1-MMP on cell surface (Mem-MT1) was biotinylated and precipitated with streptavidin beads followed by Western blotting using MT1-MMP antibody. β-actin was detected in an aliquot of total cell lysates as a loading control. (h, i) Fluorescence immunocytochemistry analyses of MT1-MMP staining on the cell surface of HO-8910PM cells cultured under normoxic or hypoxic conditions or in the presence of YC-1 (100 μM), monitored by confocal laser microscopy. Pha, phalloidin. Scale bars: 20 μm. * *P* < 0.01. (A color version of this figure is available in the online journal.)

collagen gels for 24 h under either normoxic or hypoxic condition, followed by 24 h of normoxic culture. Under neither normoxic nor hypoxic conditions, HO-8910PM cells transfected with MT1-MMP siRNA displayed obvious invasion, with invasive depth and cell numbers decreased to background levels compared with cells transfected with scrambled siRNA (Figure 4(c) and (d)). Furthermore, MT1-siRNA-treated HO-8910PM cells did not exhibit extended shapes as the control cells, and cell number did not increase compared with control cells, under both normoxic and hypoxic conditions (Figure 4(e) to (g)). Consistent with the inhibitory effects of MT1-siRNA on invasion and proliferation, similar results were obtained in the hydroxyproline assay, which showed that collagen degradation was markedly decreased by silencing MT1-MMP (Figure 4(h)).

Hypoxia induces Snail expression and nuclear translocation in HO-8910PM cells in an YC-1 sensitive manner

Although hypoxia induces MT1-MMP expression, which regulates 3D collagen invasion and degradation and HO-8910PM cell proliferation in 3D collagen, the upstream regulator of MT1-MMP is not known. We previously demonstrated that Snail mediates growth factor PDGF-BB-induced MT1-MMP expression. To study whether hypoxia affects Snail expression, HO-8910PM cells were treated with or without hypoxia in the absence or presence of YC-1, and expression of Snail was detected by RT-PCR and western blot. As shown in Figure 6(a) and (b), weak Snail expression was observed under normoxic conditions. After treatment with hypoxia, however, expressions of both mRNA and protein levels of Snail were significantly

Figure 4 MT1siRNA inhibits hypoxia-induced invasion, proliferation, and collagen degradation of HO-8910PM cells in 3D collagen via suppression MT1-MMP. (a, b) RT-PCR and Western blot analyses of MT1-MMP siRNA transfection efficiency in HO-8910PM cells under hypoxia. (c, d) 3D invasion of HO-8910PM cells, or Scrsi siRNA- or MT1-MMP siRNA-transfected cells under normoxia and hypoxia observed by phase-contrast microscopy. (e–g) HO-8910PM cells growth in 3D collagen. Red double-headed arrows demarcate cell length in collagen. Bars: 100 μm. (h) Hydroxyproline quantification. * $P < 0.01$. (A color version of this figure is available in the online journal.)

enhanced (Figure 5(a) and (b)). As expected, these increases were markedly ablated by pretreatment with YC-1. To determine localization of Snail in HO-8910PM cells, immunocytochemistry was performed to detect the subcellular distribution of Snail in HO-8910PM cells under each condition. Without hypoxia treatment, no evident Snail staining was seen (Figure 5(c)). However, during the time course of hypoxia, Snail increased gradually from 2 to 4 h, and by 16 h, Snail translocated into nuclei (Figure 5(c)). In the presence of YC-1, immunofluorescence staining of Snail

was almost invisible, indicating that hypoxia can promote Snail expression and distribution in an YC-1 sensitive manner.

Snail mediates MT1-MMP expression and 3D collagen invasion induced by hypoxia

To further investigate the relationship between Snail and MT1-MMP in hypoxia induction, Snail-specific siRNA and Snail recombinant plasmids were used to knockdown

Figure 5 Hypoxia induces snail expression and translocation to nucleus in HO-8910PM cells in an YC-1 sensitive manner. (a, b) RT-PCR and Western blot analyses of Snail in HO-8910PM cultured under normoxic or hypoxic conditions (1% O₂, or CoCl₂ 150 or 250 μ M) in the presence or absence of YC-1 (100 μ M). * $P < 0.01$. (c) Fluorescence immunocytochemistry staining of Snail (green), Phalloidin (red), and DAPI (blue) in HO-8910PM cells cultured under normoxic or hypoxic conditions in the presence or absence of YC-1 (100 μ M), monitored by confocal laser microscopy. Scale bar: 20 μ m. (A color version of this figure is available in the online journal.)

and overexpress Snail, respectively, to study its effect on MT1-MMP expression and collagen invasion. In cells transfected with Snail-specific siRNA (snsi), both Snail and MT1-MMP mRNA fell down to background levels (Figure 6(a)). Moreover, silencing of Snail significantly decreased MT1-MMP protein expression, whereas siRNA negative control-treated cells displayed clear MT1-MMP expression both at mRNA and protein levels (Figure 6(a) and (b)). Overexpression of Snail with a human Snail recombinant plasmid (pSn) in HO-8910PM cells resulted in an enhanced invasion in 3D collagen compared with empty vector-transfected HO-8910PM cells (vec) under normoxic conditions (Figure 6(c), (e), and (f)). Furthermore, under hypoxia

conditions, snsi-treated cells were unable to traverse collagenous barriers, whereas cell invasion was unaffected by transfection with scrambled siRNA negative controls (Figure 6(d) to (f)).

Hypoxia increase of HO-8910PM cell tissue invasion in vivo is dependent on Snail and MT1-MMP

To assess the role of MT1-MMP in regulating HO-8910PM cell trafficking in an *in vivo* setting, pretreated HO-8910PM cells were fluorescently labeled and cultured on the chick embryo CAM, composed of type IV collagen basement membrane and interstitial type I and III collagens

Figure 6 Snail mediates MT1-MMP expression and 3D collagen invasion under hypoxia. (a, b) RT-PCR and Western blot analyses of Snail and MT1-MMP expression in HO-8910PM cells transfected with either scrambled siRNA (scrsi) or Snail-specific siRNA (snsi) under hypoxic conditions. * $P < 0.01$. (c–f) 3D collagen invasion of HO-8910PM cells transfected with empty vector (vec) or Snail plasmid (pSn), cultured under normoxic conditions, or transfected with scrsi or snsi, cultured under hypoxic conditions. Scale bar: 100 μm . * $P < 0.01$. (A color version of this figure is available in the online journal.)

underneath. After a two-day culture period, normoxic HO-8910PM cells were found to cross the CAM surface and infiltrate the underlying stromal tissues (Figure 7(a)). However, hypoxia-treated cells displayed more significant invasion, breaking the upper CAM surface and infiltrating subjacent interstitial tissue in the embryos. Consistent with our *in vitro* findings, hypoxia-induced HO-8910PM cell invasion was blocked completely in either the presence of YC-1, or GM6001, or after silencing with MT1-MMP siRNA or Snail siRNA (Figure 7(b)).

Discussion

Hypoxia is one of the basic characteristics of solid tumors. HIF-1 α is a mediator for hypoxia during physiological and pathophysiological responses and regulates O₂ homeostasis *in vivo*.³² Under normoxia conditions, HIF-1 α is easily controlled through hydroxylation of prolyl-hydroxylase and destroyed by ubiquitinylation-mediated degradation by the von Hippel-Lindau tumor suppressor (pVHL) E3 ligase complex.³³ Under hypoxia conditions, HIF-1 α becomes stable and in combination with HIF-1 β , transfers to nuclei and activates gene expression as a transcription factor. So far, HIF-1 α has been found to regulate more than 100 low oxygen genes, such as vascular endothelial growth factor, glucose carrier 1, and MMPs.³⁴ In the present study,

we used two experimental models, 1% O₂ atmosphere and the hypoxia-mimicking chemical agent CoCl₂, to manufacture hypoxia conditions. Using both methods, HIF-1 α was increased markedly and a similar invasion and gene expression pattern of both Snail and MT1-MMP were found in HO-8910PM ovarian carcinoma cells. These data are consistent with wide expression of HIF-1 α in ovarian cancer tissue.^{35,36}

Snail is a zinc-finger transcription factor that mainly affects morphological changes during tumor progression and embryonic development.³⁷ Recently, a hypoxia response element was found in the promoter of the snail gene.³⁸ In addition, we previously found that Snail mediates MT1-MMP expression in stem cells by triggering with PDGF-bb.¹⁸ Our data presented here show that expression of Snail at both mRNA and protein levels and translocation to the nucleus are upregulated in HO-8910PM cells under hypoxia in an YC-1 sensitive manner. Overexpression of Snail with a human Snail recombinant plasmid causes an enhanced invasion in 3D matrix, while silencing Snail with Snail-specific siRNA significantly decreases MT1-MMP mRNA and protein expressions as well as invasive activities.

Current studies indicate that tissue invasive processes may depend on protease selection, which is regulated by both the motile cell population characteristics and

Figure 7 Hypoxia increases HO-8910PM cell tissue invasion *in vivo* reliant on Snail and MT1-MMP. HO-8910PM cells treated with hypoxia, in the presence or absence of YC-1 (100 μ M) or GM6001 (25 μ M), or treated with siRNAs were fluorescently labeled with green fluoresbrite carboxylate microspheres and then seeded atop the chorioallantoic membrane (CAM) of 11-day-old chick embryos for two days. Frozen sections were stained with antitype IV collagen antibody (red) and fluorescent images were captured by fluorescence microscopy, with white arrow heads indicating invasive HO-8910PM cells in the embryo tissues and red arrows on the right of the panel indicating the surface of CAM. Invasion is quantified as the number of HO-8910PM cells that cross the CAM surface and infiltrate the underlying stromal tissues (mean $\pm$ SEM, $n = 3$). * $P < 0.01$. Scale bars: 100 μ m. (A color version of this figure is available in the online journal.)

intervening ECM structural properties.³⁹ Previously we used a 3D collagen model and chick embryo, which is close to the *in vivo* mammalian environment, and found that MT1-MMP plays a key role in promoting mesenchymal stem cells to traffic through both matrix barriers.²⁶ Our present data demonstrate that when HO-8910PM cells are confronted with hypoxia, cells upregulate MT1-MMP expression on the mRNA level, subsequently increasing MT1-MMP biosynthesis in endoplasmic reticulum and translocation to the cytomembrane, where degrading cellular matrix and thereby generating prerequisite space for cell proliferation and metastasis. Inhibition of MT1-MMP by GM6001 or specific siRNA significantly decreased HO-8910PM cell invasion and proliferation in the 3D matrix, further identifying MT1-MMP as a key regulator/effector in hypoxia-induced invasion and proliferation.

In the present study, MT1-MMP on cell surface was increased under hypoxia, as detected by confocal laser

scanning microscopy as well as biotinylation assay, consistent with a report showing that hypoxia promoted traffic of MT1-MMP from cytoplasm storage pools to the cell surface in SK-3rd TICs.⁴⁰ This increase on the surface of hypoxic HO-8910PM cells likely is resulted, at least in part, by enhancement of protein synthesis, since significant MT1-MMP staining was found around endoplasmic reticulum, which is the location for protein translation.

In conclusion, our data presented here indicate that hypoxia induces Snail expression and further upregulates MT1-MMP expression and activity, thereby enhancing ovarian cancer cell invasive ability in 3D matrix.

Authors' contributions: LS, PL, ZQ: Conception and design, performed the experiment, data analysis and interpretation.

YL, L-L D: Performed the experiment, data analysis and interpretation.

CL: Conception and design, performed the experiment, data analysis and interpretation, manuscript writing, and provided financial support.

LS, PL, ZQ, and YL made equal contribution to this work.

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