

## A role for endothelial-derived matrix metalloproteinase-2 in breast cancer cell transmigration across the endothelial-basement membrane barrier

Hamed Kargozaran · Sarah Y. Yuan · Jerome W. Breslin · Katherine D. Watson · Nathalie Gaudreault · Alison Breen · Mack H. Wu

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**Abstract** Invasive cancer cells utilize matrix metalloproteinases (MMPs) to degrade the extracellular matrix and basement membrane in the process of metastasis. Among multiple members of the MMP family, the gelatinase MMP-2 has been implicated in the development and dissemination of malignancies. However, the cellular source of MMP-2 and its effect on metastatic extravasation have not been well characterized. The objective of this study was to test the hypothesis that active MMP-2 derived from endothelial cells facilitated the transmigration of breast cancer cells across the microvascular barrier. Gelatin zymography was used to assess latent and active MMP-2 production in conditioned media from MDA-MB-231 human breast cancer cells, human lung microvascular endothelial cells (HLMVEC) and co-culture of these two cells. Transmigrated cancer cells were measured during MMP-2 knock-down with siRNA and pharmacological inhibition of MMP activity with OA-HY. The results showed consistent MMP-2 secretion by the HLMVECs, whereas a low level production was seen in the MDA-MB-231 cells. Inhibition of MMP-2 expression or activity in HLMVECs significantly attenuated the transmigration of MDA-MB-231 cells across an endothelial monolayer barrier grown on a reconstituted basement membrane. The data provide evidence supporting a potential role for the endothelial production of MMPs in promoting cancer cell extravasation. We suggest that the interaction between malignant cells and peritumoral benign

tissues including the vascular endothelium may serve as an important mechanism in the regulation of tumor invasion and metastasis.

**Keywords** Breast cancer · Metastasis · Microvascular barrier

### Introduction

Breast cancer is the most frequently diagnosed malignancy in women. In the United States a woman's lifetime risk of developing breast cancer is one in eight [1]. As with many other malignancies, most cases of breast cancer death can be attributed to distant metastasis, a process characterized by the growth of the primary tumor, vascular or lymphatic invasion, transport survival, extravasation across the endothelium-matrix barrier, and eventual establishment of metastatic lesions at a distant site. The mechanisms underlying this process involve complex gene regulation and molecular interactions occurring in both malignant cells and normal tissues. Matrix metalloproteinases (MMPs) play an important role in metastatic progression based on their abilities to stimulate tumor cell growth and facilitate breakdown of the extracellular matrix and basement membrane [2, 3]. Furthermore, evidence indicates that MMP activation is associated with the production and release of growth factors and chemotactic factors that promote tumor development and metastasis [4, 5]. Among multiple MMPs, the gelatinase MMP-2 has been well recognized for its role in various malignant processes. The primary substrate of MMP-2 is collagen type IV, a major component of the basement membrane that forms a barrier to tumor invasion and extravasation. Recent studies have shown that inhibition of MMP-2 activity reduces the

H. Kargozaran · S. Y. Yuan · J. W. Breslin · K. D. Watson · N. Gaudreault · A. Breen · M. H. Wu (✉)

Department of Surgery, Division of Research,  
University of California Davis School of Medicine,  
4625 2nd Avenue, Room 3006, Sacramento, CA 95817, USA  
e-mail: macwu@ucdavis.edu

metastatic potential of malignant cells both in vitro and in vivo, and MMP-2 downregulation leads to decreased tumor cell invasion across matrigel [6–9]. In animal studies, MMP-2 inhibition decreases metastatic burden in the bone and lung [10–12].

In addition to the extracellular matrix, the microvascular endothelium is important as it serves as a selective barrier to the extravasation of cancer cells from the circulation [13]. The exact mechanism of transvascular migration is largely unknown, but likely involves the interaction between malignant cells and the endothelium that leads to an opening of endothelial barrier coupled with degradation of basement membrane and other components of the perivascular matrix, allowing the malignant cells to reach the target tissue [14]. Whether MMP-2 plays a role in this process has not been evaluated.

In this study, we focused on MMP-2 with respect to its effect on the transmigration of MDA-MB-231 human breast cancer cells across the human lung microvascular endothelial cell (HLMVEC) barrier. We employed a co-culture model and assessed the relative contribution of MDA-MB-231 vs. HLMVEC to the overall MMP-2 secretion and activity. We also examined the role of MMP-2 in the transendothelial migration of cancer cells by using a pharmacological approach that inhibits MMP activity, as well as through siRNA silencing of MMP-2 gene expression.

## Materials and methods

### Reagents and cell culture media

MMP-2 inhibitor I (OA-HY) was purchased from Calbiochem (San Diego, CA) and dissolved in dimethyl sulfoxide (DMSO). The DMSO level was kept under 0.1% in all experimental media. Human MMP-2 SMARTpool Plus reagent and non-targeting siControl RNA were purchased from Dharmacon (Lafayette, CO). Oligofectamine reagent, and Live-Dead assay were purchased from Invitrogen (Carlsbad, CA). Human breast cancer cells (MDA-MB-231), a highly invasive breast cancer cell line derived from pulmonary metastasis, were obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium containing 10% FBS (Mediatech, Herndon, VA). HLMVECs were purchased from American Type Culture Collection and grown in EBM-2 medium 5% FBS (Cambrex, Walkersville, MD).

### Gelatin zymography

Gelatin zymography assay kits were purchased from Invitrogen (Carlsbad, CA). To analyze MMP-2 production,

MDA-MB-231 cells and HLMVECs ( $3 \times 10^5$ ) were seeded on 35 mm culture plates in complete growth media. One day after seeding, the cells were washed twice with warm PBS and serum free media was added and allowed to condition for 12 h. 20  $\mu$ l samples from the conditioned media were subjected to gelatin zymography as instructed by the manufacturer. These samples were pelleted by centrifugation. The supernatants were mixed with 2 $\times$  SDS buffer and fractionated on a 10% gelatin containing zymography gel under nonreducing conditions. After two washes with zymogram renaturing solution, the gels were incubated overnight in developing buffer at 37°C. The gels were then stained with colloidal blue for 6 h and de-stained with water for 12 h. Clear bands correlating with areas of gelatinase activity were visualized and imaged with Alpha Innotech Imaging software (San Leandro, CA). The bands were quantified using densitometry.

### Transwell invasion assay

After serum starvation for 24 h, MDA-MB-231 cells were treated with 5  $\mu$ M of OA-HY, a MMP-2 inhibitor [15, 16], for 30 min at room temperature. Control cells were treated with an equivalent concentration of the vehicle DMSO. The cells were washed twice with warm PBS, trypsinized and suspended in warm HBSS containing 5  $\mu$ M of calcein-AM (Invitrogen, Carlsbad, CA). After 30 min of incubation, the stained cells were washed twice with HBSS. A total of  $10^4$  cells were suspended in 300  $\mu$ l of serum free media and added to the top chamber of 8  $\mu$ m pore transwell coated with reconstituted basement membrane (Millipore, Billerica, MA). About 500  $\mu$ l of EBM-2 media containing 10% FBS was added to the bottom chamber. The cells were incubated for 12 h and allowed to migrate. Remaining cells on the upper surface of the membrane were gently removed using a cotton swab. A Zeiss Axiovert 200M fluorescent microscope (Thornwood, NY) was used to image the cells that had migrated. Cells from five random fields (0.6 mm<sup>2</sup> area) were counted.

### Co-culture and transendothelial migration assay

HLMVEC ( $10^5$ ) were seeded on the top chamber of the aforementioned transwell apparatus and grown to confluence as previously described [17, 18]. The medium was replaced on a daily basis. Live-Dead assays were performed 3 days after seeding to ensure endothelial cell viability and confluence. The endothelial layer was treated with OA-HY (5  $\mu$ M) or DMSO as vehicle control for 30 min at room temperature. MDA-MB-231 cells were treated with the same concentration of OA-HY or vehicle

and labeled with Calcein-AM as described above. All treated cells were washed twice with warm PBS. MDA-MB-231 ( $10^4$ ) cells suspended in 300  $\mu$ l of serum free EBM-2 media were added to the endothelial layer in the upper chamber. EBM-2 media containing 10% FBS (500  $\mu$ l) was added to the lower chamber. At the end of the experiment, non-migrating cancer cells and the endothelial layer on the upper surface were removed using a cotton swab. The cells that migrated were imaged and counted as described above.

#### siRNA gene silencing

HLMVECs ( $3 \times 10^5$ ) were seeded on gelatin coated 35 mm plates. Oligofectamine 2000 (Invitrogen) was used to transfect cells with MMP-2 siRNA as per the manufacturer's instructions. Briefly, 20 nM of MMP-2-specific siRNA or non-targeting siRNA (Dharmacon, Lafayette, CO) was mixed with 8  $\mu$ l of oligofectamine in Opti-MEM and allowed to incubate for 20 min. Then the siRNA/oligofectamine mixture was added to HLMVECs. After 4 h of incubation, EBM-2 media was added to the culture dishes. The siRNA transfection efficiency was verified using the BLOCK-iT fluorescent oligo assay (Invitrogen) and the data (not shown) indicated approximately 70% cells were transfected. A preliminary zymographic analysis of the conditioned media showed a maximal inhibition of MMP-2 at 3 days post-transfection. In a separate set of experiments, HLMVECs ( $10^5$ ) were transfected with either MMP-2 siRNA or non-targeting siRNA. About 3 days post transfection, MDA-MB-231 ( $10^4$ ) cells were added for co-incubation with the HLMVEC for 10 h in serum free media. About 20  $\mu$ l samples from the co-culture conditioned media were collected for gelatin zymography. For quantification of transendothelial migration, siRNA transfected endothelial cells were seeded on the transwell membranes and grown to confluence. After 3 days,  $10^4$  of labeled MDA-MB-231 cells were added on top of the endothelial cells and allowed to migrate for 12 h in serum free media. The migrated cancer cells were then imaged and counted.

#### Statistical analysis

For each experimental intervention, the values of microscopic readings were averaged from multiple groups and normalized to the basal values obtained before the intervention. All data are presented as % of control in Mean  $\pm$  SE, with the  $n$  number representing separate experiments using different dishes of cells. Analysis of variance (ANOVA) was used to evaluate the significance of inter-group differences. A value of  $P < 0.05$  was considered significant for the comparisons.

## Results

### MMP-2 activities in cancer and endothelial cells

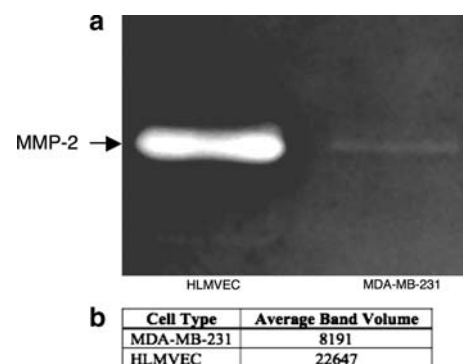
Serum free conditioned media collected from an equivalent number of cells were analyzed for the presence of active MMP-2 using gelatin zymography. Minimal gelatinase activity was observed in MDA-MB-231 cells. In contrast, a considerable amount of MMP-2 was detected in conditioned media from HLMVEC under the basal condition (Fig. 1).

### MMP-2 inhibition and basement membrane invasion

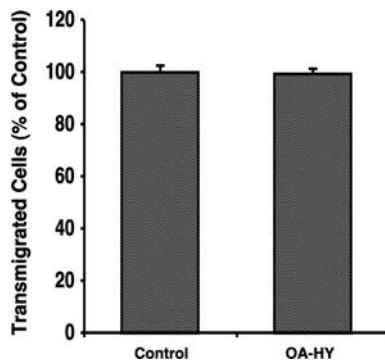
We next assessed the role of MMP-2 in the migration of MDA-MB-231 cells across basement membrane coated Transwell inserts. Zymographic analysis of MMP-2 activity from conditioned media of MDA-MB-231 cells revealed a low level of gelatinase activity in the control group. Inhibition of MMP-2 activity by treating the MDA-MB-231 cells with OA-HY did not significantly alter their ability to invade the basement membrane, as the number of cancer cells that cross the reconstituted basement membrane in the OA-HY-treated group was similar to that in the vehicle (0.1% DMSO)-treated control group (Fig. 2).

### MMP-2 inhibition and transendothelial migration

We compared the number of MDA-MB-231 cells that migrated across HLMVEC monolayers grown on basement membrane-coated Transwell surface. In the control group, MDA-MB-231 cells treated with vehicle (0.1% DMSO) were added to HLMVEC monolayers that were also treated with the vehicle. Treatment of the cancer cells with OA-HY did not significantly change their transendothelial



**Fig. 1** Differential MMP-2 secretion by HLMVEC and MDA-MB-231 cells. Conditioned media from equivalent number of HLMVEC and MDA-MB-231 cells were analyzed for MMP-2 activity using gelatin zymography. (a) HLMVEC showed a considerable amount of latent MMP-2 as compared to MDA-MB-231 cells. (b) Band volumes representing MMP-2 gelatinase activity were quantified using densitometry and averaged.  $N = 3$  for both groups



**Fig. 2** MMP-2 inhibition does not affect MDA-MB-231 cell migration across basement membrane. MDA-MB-231 cells were treated with the MMP-2 inhibitor OA-HY. No significant difference in migration across basement membrane coated inserts was seen between cancer cells treated with OAHY and control cells.  $N = 6$  for both groups

migration as compared to control. However, treatment of the endothelial cells with the same inhibitor resulted in a significant attenuation of cancer cell transendothelial migration. Treating both cells with OA-HY reduced transendothelial migration to a similar extent as treating only HLMVEC with OA-HY (Fig. 3).

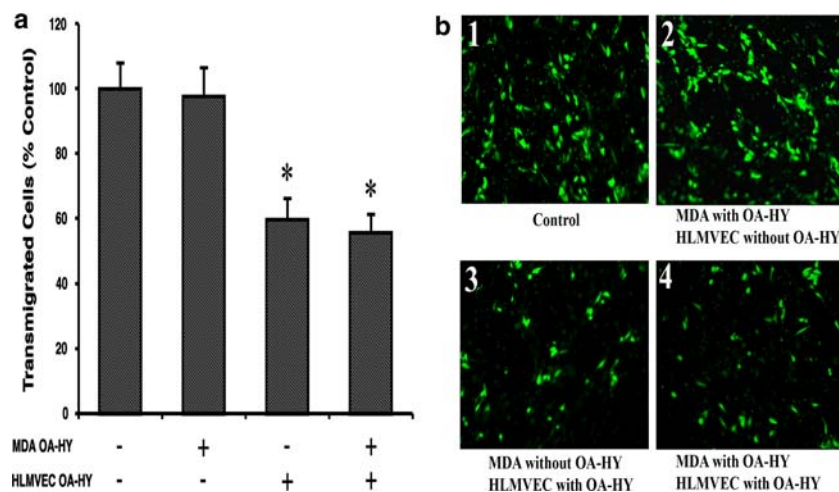
#### MMP-2 knockdown and transendothelial migration

To provide further evidence for the involvement of endothelial-derived MMP-2 in cancer cell transmigration, we measured MMP-2 activity and cancer cell transmigration in endothelial cells subjected to siRNA knockdown of MMP-2

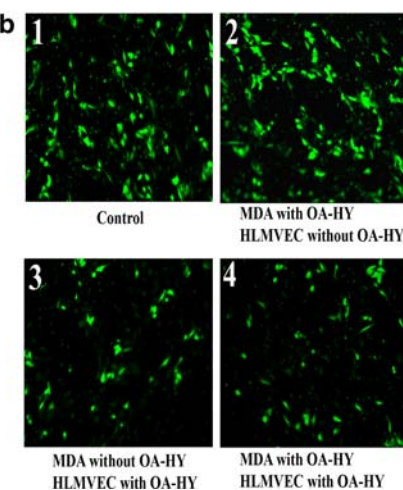
expression. HLMVEC treated with a non-targeting RNA sequence served as control. Samples of conditioned media of HLMVEC seeded in transwell chambers were taken just prior to starting the migration assay, and confirmed a significant reduction in the MMP-2 level following siRNA transfection (Fig. 4A). We also assessed MMP-2 from the co-culture conditioned media. Interestingly, co-culture of HLMVEC and MDA-MB-231 cells caused the appearance of a 62-kD band representing the cleaved, active form of MMP-2 (Fig. 4B). This band was not present when HLMVEC were treated with MMP-2 siRNA prior to co-culturing with MDA-MB-231, indicating the possibility that the endothelial cells served as an important source of this active form of MMP-2. In agreement with this, siRNA knockdown of endothelial MMP-2 also caused a significant decrease in the transendothelial migration of MDA-MB-231 cells (Fig. 5).

#### Discussion

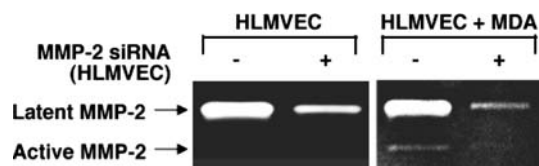
This study provides supporting evidence for the potential involvement of endothelial cell-derived MMP-2 in the transmigration of breast cancer cells across the endothelium-matrix barrier. Our data show that HLMVECs secrete more MMP-2 than breast cancer cells in culture. In addition, siRNA knockdown of MMP-2 gene expression and pharmacological inhibition of MMP activity in endothelial cells significantly attenuate transendothelial migration of cancer cells.



**Fig. 3** The role of MMP-2 in transendothelial migration of MDA-MB-231 cancer cells. **(a)** No significant difference in transendothelial migration was observed with treatment of cancer cells with OAHY. However, treatment of endothelial cells with OAHY significantly decreased the transendothelial migration of cancer cells.  $*P < 0.05$  versus both groups in which HLMVEC were not treated with OAHY.



$N = 6$  for all groups. **(b)** The panels show representative transmigrated cancer cells, labeled with calcein AM, in the four experimental groups: 1- control 2- cancer cells treated with OAHY/Control HLMVEC 3- Control cancer cells/OAHY treated HLMVEC 4- both cell types treated with OAHY



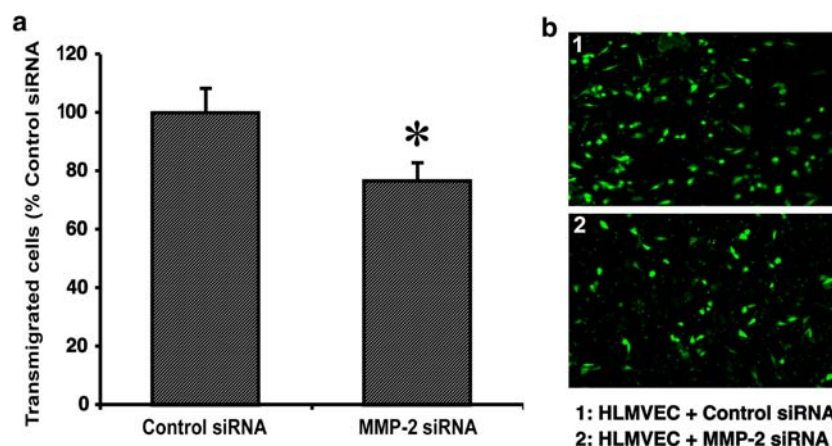
**Fig. 4** Silencing of MMP-2 expression and activation. The two left lanes in the zymogram respectively show the MMP-2 bands obtained from condition media of HLMVEC that were treated with siCONTROL RNA (–) or MMP-2 siRNA (+). In the zymogram on the right, HLMVEC were transfected and grown in the same fashion and then co-cultured for 10 h with MDA-MB231 cancer cells, after which conditioned medium was collected. The latent and active MMP-2 bands correspond to 72 and 62 kDa, respectively. The zymograms shown are representative of three separate experiments

Different types of cancers tend to metastasize to distinctive target tissues. Although the mechanisms that determine the route and tissue selectivity of metastasis remain to be identified, the first step of blood-borne metastasis requires circulating malignant cells to be arrested at the surface of the microvascular endothelium, followed by transmigration across this selective barrier. Because the lung is a well-recognized target of breast cancer metastasis and is in fact where MDA-MB-231 cells were isolated from [19], we chose the microvascular endothelial cell of human lung origin as the primary model to assess breast cancer cell invasion and extravasation.

The role of MMPs in tumor development and metastasis has been studied under various conditions. Unlike other studies that have attempted to identify stimuli that increase the tumor cell-dependent MMP activity [12, 20–22], we investigated the possibility that metastatic cells take advantage of MMPs produced by non-tumor cells at sites of metastasis. Gelatin zymography revealed a low level of

MMP-2 secretion in conditioned media from MDA-MB-231 cells. This is consistent with previous studies reporting basal MMP-2 expression in cultured MDA-MB-231 cells ranging from undetectable to very low levels [23–25], as well as the notion that epithelial carcinomas, such as breast cancer, typically produce minimal amounts of MMP-2 [26, 27]. The low level of MMP-2 secretion from MDA-MB-231, along with the evidence that inhibition of MMP-2 in these cells did not alter their ability to migrate across an artificial basement membrane, argues against the predominant role of malignant cell-derived MMP-2 in matrix degradation during cancer invasion or extravasation under the culture condition. In contrast, HLMVEC produced a significantly greater amount of MMP-2 than the cancer cells, and inhibition of endothelial MMP-2 markedly reduced transendothelial migration. The data suggest that cancer cells may be able to interact with the vascular endothelium at sites distal from the original tumor, promoting the dissemination of malignancy. In support of this hypothesis, others have shown that MMP-2 produced by non-malignant cells is involved in the earlier steps of metastasis, as the MMPs produced by peritumoral stromal cells, such as fibroblasts, play a major role in the remodeling of peritumoral extracellular matrix that allows the escape of cancer cells from the primary tumor [28, 29]. The interaction of cancer cells with fibroblasts upregulates the cell surface glycoprotein extracellular matrix metalloproteinase inducer (EMMPRIN), which in turn stimulates the production of MMPs from peritumoral stroma and leads to increased metastatic potential in several types of malignancies [30, 31].

The current study provides direct evidence for an interactive role of pulmonary microvascular endothelial



**Fig. 5** Silencing of MMP-2 expression in HLMVEC decreases transendothelial migration of MDA-MB-231 cancer cells. (a) Treatment of HLMVEC with MMP-2 siRNA caused a significant decrease in transendothelial migration of cancer cells, as compared with treatment with a non-targeting, control RNA sequence.

\*Indicates  $P < 0.05$ , MMP-2 siRNA versus control.  $N = 6$  for both groups. (b) The panels show representative transmigrated cancer cells labeled with calcein AM; (1) cancer cells that migrated across control HLMVEC, (2) HLMVEC transfected with MMP-2 siRNA

cells in breast cancer cell extravasation that may be facilitated by the endothelial-derived MMP activity. Conditioned media from either HLMVEC or MDA-MB-231 cells only showed the presence of latent MMP-2. However, both active and latent forms of MMP-2 were present in conditioned media when these two cell types were co-cultured. These findings suggest an interaction between endothelial and cancer cells that promotes MMP-2 activation. Interestingly, active MMP-2 was not seen in co-cultures in which siRNA transfection was used to inhibit MMP-2 expression in HLMVEC. Thus, endothelial cells appear to be a major source for generating active MMP-2 in response to cancer cell stimulation. The interaction between tumor cells and the endothelium may elevate the level of active MMP-2 via the cell surface protein MT1-MMP. Located on the cell surface, pro-MMP-2 forms a ternary complex with membrane-type metalloproteinase-1 (MT1-MMP) and tissue inhibitor of metalloproteinase-2 (TIMP-2). This complex causes the cleavage of the amino-terminal propeptide and formation of active MMP-2 [32]. Several malignancies including breast carcinomas express high levels of MT1-MMP [33, 34]. Activation of pro-MMP-2 secreted by peritumoral cells has been localized to breast cancer cell surface and is believed to be via MT1-MMP [35]. We identified the presence of MT1-MMP in the MDA-MB-231 cells using immunoblotting (Data not shown). Other investigators have also shown the expression of MT1-MMP in MDA-MB-231 cells [33, 36].

Further studies are required to characterize the specific effect of MMP-2 and its mechanism of action on cancer invasion and metastasis. We speculate that MMP-2 contributes to the breakdown of endothelial barrier and the underlying matrix bed, based on that type IV collagen, a major component of the basement membrane, is the primary substrate of MMP-2 [37]. It is possible that MMP-2 directly, or indirectly via complex signaling processes, promotes matrix network degradation and endothelial junction opening, allowing cancer cells to escape from the circulation and enter the target tissue [13, 14, 38–41]. However, MMP-2 may not be the only member of the metalloproteinase family that participates in this process. The data that pharmacological inhibition with OA-HY caused a greater reduction in transendothelial migration than did MMP-2 siRNA indicates the potential involvement of other mechanisms. The discrepancy could result from the multiple modes of action of the MMP inhibitor. OA-HY is a hydroxamate derivative of oleic acid that has been shown to inhibit multiple MMPs, including MMP-1, MMP-3, and MMP-9 [15, 42, 43]. Although we did not detect MMP-9 activity in our co-culture experiments, we could not rule out the possibility that other MMPs were affected during the drug treatment, which complicated the

results. In addition to MMPs, other proteolytic enzymes involved in cancer metastasis might be affected by oleic acid derivatives as well. Possible candidates include plasmin and urokinase plasminogen activator (uPA) which have been shown to affect cancer growth, extracellular matrix degradation and metastasis [44]. Oleic acid modulates uPA-plasmin interaction, inhibits proteolytic activity of plasmin, and interferes with plasmin-mediated pro-MMP3 activation [43]. Moreover, the discrepant inhibitory potency of the chemical agent and siRNA approach might be attributable to the complex regulatory mechanisms at multiple levels. Within this context, evidence is emerging that a large family of oncogenes and suppressor genes regulates breast cancer metastasis [45]. A recent study shows that breast cancer metastasis suppressor 1 represses transcription by forming complexes with retinoblastoma-binding protein 1 and the mSin3 histone deacetylase complex in MDA-MB-231 cells [46]. Whether this negative regulatory pathway is involved in MMP-mediated cancer cell extravasation and matrix remodeling remains an interesting question.

In summary, our data suggests an active role for the lung microvascular endothelial cells in regulating breast cancer cell invasion and extravasation. Endothelial-derived MMP-2 contributes, at least partially, to the transmigration of malignant cells across the endothelium-matrix barrier. The findings indicate the potential importance of non-tumor cells in the metastatic process. Although most current chemotherapeutic agents are targeted against malignant cells, perhaps a role does exist for targeting factors derived from benign tissues that propagate cancer metastasis. Further understanding of the molecular basis underlying the interaction between malignant and benign cells would lead to improved treatment options.

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