Roles of the Cyclooxygenase 2 Matrix Metalloproteinase 1 Pathway in Brain Metastasis of Breast Cancer*

Kerui Wu‡, Koji Fukuda‡, Fei Xing‡, Yingyu Zhang‡, Sambad Sharma‡, Yin Liu‡, Michael D. Chan‡, Xiaobo Zhou‡, Shadi A. Qasem§, Radhika Pochampally‡, Yin-Yuan Mo*, and Kounosuke Watabe‡

From the ‡Department of Cancer Biology, Wake Forest University School of Medicine, Winston Salem, North Carolina 27157, §Cancer Institute, University of Mississippi Medical Center, Jackson, Mississippi 39216, and ¶Cancer Research Institute, Kanazawa University, Kanazawa 920-0934, Japan

Background: Mechanism of brain metastasis of breast cancer is poorly understood.
Results: Inflammatory factors secreted from cancer cells degrade tight junction of BBB and stimulate the tumor-microenvironment cross-talk.
Conclusion: COX2-prostaglandins-MMP1 pathway promotes brain metastasis by tampering with BBB and up-regulating CCL7 in astrocytes for the growth of tumor initiating cells.
Significance: The COX2-prostaglandins-MMP1 pathway may serve as a novel therapeutic target for brain metastasis.

Brain is one of the major sites of metastasis in breast cancer; however, the pathological mechanism of brain metastasis is poorly understood. One of the critical rate-limiting steps of brain metastasis is the breaching of blood-brain barrier, which acts as a selective interface between the circulation and the central nervous system, and this process is considered to involve tumor-secreted proteinases. We analyzed clinical significance of 21 matrix metalloproteinases on brain metastasis-free survival of breast cancer followed by verification in brain metastatic cell lines and found that only matrix metalloproteinase 1 (MMP1) is significantly correlated with brain metastasis. We have shown that MMP1 is highly expressed in brain metastatic cells and is capable of degrading Claudin and Occludin but not Zo-1, which are key components of blood-brain barrier. Knockdown of MMP1 in brain metastatic cells significantly suppressed their ability of brain metastasis in vivo, whereas ectopic expression of MMP1 significantly increased the brain metastatic ability of the cells that are not brain metastatic. We also found that COX2 was highly up-regulated in brain metastatic cells and that COX2-induced prostaglandins were directly able to promote the expression of MMP1 followed by augmenting brain metastasis. Furthermore, we found that COX2 and prostaglandin were able to activate astrocytes to release chemokine (C-C motif) ligand 7 (CCL7), which in turn promoted self-renewal of tumor-initiating cells in the brain and that knockdown of COX2 significantly reduced the brain metastatic ability of tumor cells. Our results suggest the COX2-MMP1/CCL7 axis as a novel therapeutic target for brain metastasis.

Brain metastasis of breast cancer occurs in up to 30% of patients, which causes serious effects on mobility and mortality (1). The rate of brain metastasis is increasing with the recent advancement of chemotherapy because most therapeutic agents are unable to reach brain and tumor cells ironically find the organ as a sanctuary. The median survival time of patients with brain metastasis is less than one year (1). Despite this clinical importance, the pathological mechanism of brain metastasis is as yet poorly understood. Metastatic tumor cells must first breach the blood-brain barrier (BBB), which acts as a selective interface between the peripheral circulation and the central nervous system. BBB is a unique anatomical structure that is mainly constructed by tight junctions between the brain endothelial cells and pericytes that are embedded in the basement membrane of the endothelial cells (2–8). The tight junction confers low paracellular permeability and high electrical resistance, making the barrier function 50–100 times tighter than peripheral microvessels (9). This structure is further strengthened by astrocytes that play a decisive role in the maintenance of the barrier properties of the brain microcapillary endothelial cells in controlling the flow of ions, nutrients, and cells into the brain. Rolling leukocytes are known to cross the inflamed BBB followed by entry into the CNS (3); however, whether tumor cells use the similar mechanism to penetrate the BBB is still under investigation (10–13).

The metalloproteinase family consists of >25 variants, and some of the genes are known to play critical roles in invasion, intra- and extravasation, and angiogenesis in tumor metastases (14, 15). MMP1 has also been implicated in cancer progression and metastasis (11). Despite promising hope in metalloproteinases as therapeutic targets, use of general inhibitors of metalloproteinases in clinical trials did not yield effective outcome (16), indicating that more detail and systematic analysis

* This work was supported, in whole or in part, by National Institutes of Health Grant R01CA124650, R01CA129000, and R01CA173499 (to K.W.). This work was also supported by Tumor Tissue Core facility of the Comprehensive Cancer Center of Wake Forest University.

† Both authors contributed equally to this work.

‡ To whom correspondence should be addressed: Dept. of Cancer Biology, Wake Forest University School of Medicine, Winston Salem, NC 27157. Tel.: 336-716-0231; Fax: 336-716-0255; E-mail: kwatabe@wakehealth.edu.
of the role of metalloproteinases is necessary. Interestingly, brain ischemia is reported to stimulate the permeability of the BBB by promoting disruption of tight junction complex via activation of MMP2 and -9 (17–20). In other studies, MMP3, -8, and -9 were shown to be able to cleave Occludin, a key protein controlling the tight junction (21–23). In CNS leukemia, MMP2 and MMP9 secreted by leukemic cells were found to promote the disruption of the tight junction proteins including ZO-1 and Claudin-5, which then increased paracellular permeability of leukemic cells across the BBB (10, 24, 25). In breast cancer, MMP2 has been shown to be involved in brain metastasis, although the exact role of this metalloproteinase on penetrating BBB is not clear (26). Because each metalloproteinase has a different enzymatic activity and substrate, such as the collagenases (MMP-1, -8, and -13), the gelatinases (MMP-2 and -9), the stromelysins (MMP-3, -10, and -11), and a heterogeneous group containing matrilysin (MMP-7), metallo-elastase (MMP-12), enamelysin (MMP-20), endometase (MMP-26), and epilysin (MMP-28), it is imperative to systematically examine and clarify the roles of these metalloproteinases in the process of BBB penetration in order to identify specific therapeutic targets for brain metastasis.

Even when tumor cells successfully penetrate and invade the brain, they must adapt themselves to a totally different microenvironment from that of the primary site. According to recent cancer stem cell theory, metastatic cells should possess stem-like or tumor-initiating cell properties and generate a particular niche at the distant metastatic sites (27, 28). How breast tumor initiating cells create or adapt a specific niche for their self-renewal in the brain is an intriguing question for therapeutic and prognostic purposes. Astrocytes are the most abundant cell type in the brain, and several lines of evidence indicate that astrocytes are activated by tumor cells and in turn support the growth of metastatic cells (29). Therefore, it is of paramount interest to dissect the role of the activated astrocytes in generating the niche for metastatic breast cancer. To address these critical questions, we examined the correlations of the expression of all metalloproteinases with brain metastasis of breast cancer patients and also the expression profile of these genes in brain metastatic tumor cell lines followed by in vivo verification. We found that MMP1 plays a critical role in BBB penetration and that COX2-mediated prostaglandin promotes proliferation of tumor initiating cells by activating tumor associated astrocytes followed by secretion of CCL7.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture**—Human breast carcinoma cell line, MDA-MB-231, was purchased from American Type Culture Collection (ATCC). 231LM, 231BrM-2a, CN34, and CN34-BrM2c cell lines were kindly provided by Dr. Joan Massagué (Memorial Sloan-Kettering Cancer Center). Luciferase-labeled cells were generated by infecting the lentivirus carrying the firefly luciferase gene. The immortalized mouse brain microvascular endothelial cell (mBMEC) was a generous gift from Dr. Isaiah J. Fidler (MD Anderson Cancer Center). MDA-MB-231 and its variant cells were cultured in DMEM medium supplemented with 10% FBS and antibiotics. CN34 and CN34-BrM2c cells were cultured in Medium199 supplemented with 2.5% FBS, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 20 ng/ml EGF, 100 ng/ml cholera toxin, and antibiotics. E6/E7/hTERT, immortalized human astrocyte cells (UC-1), was a kind gift from Dr. Russell Piper (University of California San Francisco), and they were cultured in DMEM with 10% FBS. mBMECs were maintained at 8% CO2 at 33 °C in DMEM with 10% FBS, 2 mM l-glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids, and 1% vitamin mixture. MDA-MB-231 and 231BrM-2a were authenticated by conducting Affymetrix expression array analysis, and they were routinely tested for the absence of mycoplasma.

**Isolation of Tumor Initiating Cell Population by Magnetic-activated Cell Sorting (MACS)**—Tumor initiating cells were isolated by the MACS system (Miltenyi Biotec) using antibodies to CD24 (Stem Cell Technologies), CD44 (Biolegend), and ESA (GeneTex). Briefly, cells were treated with trypsin and suspended in MACS buffer (PBS with 1 mM EDTA and 0.1% FBS). The cells were labeled with biotin-conjugated anti-CD24 and allopbyocyanin-conjugated anti-CD44 at 4 °C for 15 min in the MACS buffer. Cells were then washed and further incubated with anti-biotin micro beads followed by sorting out the CD24high cells by using the MACS column. Next, the CD24low fraction was incubated with anti-allophycocyanin micro beads, and CD24low/CD44high was collected by passing through the MACS column. Cells were then incubated with biotin-conjugated anti-ESA followed by incubation with anti-biotin micro beads. Finally, CD24low/CD44high/ESAhigh cells (tumor initiating cells) were isolated by using the MACS column. Isolated tumor initiating cell population was confirmed by FACS.

**Trans Brain Endothelial Assay**—For the trans brain endothelial assay, we used a 24-well cell culture insert, microscopically transparent polyester membrane of 6-mm diameter and 3.0-μm pore size. Astrocytes cells (UC-1) were first seeded on the underside of the transwell for 12 h, and mBMECs were then seeded on the top side of the membrane followed by incubation for 1 day. Breast cancer cells labeled with GFP were then seeded into the transwell insert. After 24 h, GFP labeled cells that had migrated through the mBMEC and astrocytes were counted under a fluorescent microscope.

**Trans-endothelial Electrical Resistance (TEER) and Permeability Assays**—TEER was assessed post-treatment in confluent mBMECs monolayers using an EVOM™ Epithelial Voltmeter (World Precision Instruments, Sarasota, FL). Briefly, Transwell-Clear inserts as described above were seeded with cancer cells followed by the indicated treatment, washed twice with PBS, and transferred into an Endohm™-24 TEER measurement chamber. Serum/antibiotic-free DMEM was used as the electrolyte solution at room temperature. To calculate TEER, baseline resistance reading from a Transwell-Clear insert “without” cells was subtracted from the resistance reading for each condition with cells. For permeability assay, the same transwell chambers with astrocytes and endothelial cells in phenol red-free DMEM were used. After the confluent endothelial monolayers were formed, medium was replaced with conditional medium, and the wells were further incubated for 24 h. The chambers were then washed with PBS three times. Evans blue (EB) albumin was then added to the luminal chamber to a final concentration of 0.5% in phenol red-free DMEM. After 24 h,
100 μl of media was removed from the abluminal chamber for measurement of absorbance at 600 nm (A_{600}). The clearance of EB-albumin was determined by plotting the absorbance to standard curve of EB-albumin.

Real-time PCR and Western Blotting—qRT-PCR was performed using CFX96Touch Real-time PCR detection system (Bio-Rad) and the SYBR Green qPCR kit (Fermentas). Primers used in this study were: COX2-F, CAAAAGCTGGGGACCTTTCT; COX2-R, CCATCTTTTGGAAAGGCCGAG; MMP1-F, GAGCTCAACTTCCGGGTAGA; MMP1-R, CCAAAAAGCTGTGAGACAGTA; CCL7-F, CAGCCTCTGCTTAGGGATTTTT; CCL7-R, GCCATTCTGTGTCTGCTGCT; Nanog-F, CTCCACATCTGACCTGCACGC; Nanog-R, CATTGGAAGGTTCCCGATTCGG. Western blotting was carried out using the same methods as previously described (30). The COX2 and Nanog antibodies were purchased from Cell Signaling Co. The Claudin-5 and Occludin antibodies were obtained from Abcam. The ZO-1 antibody was purchased from Invitrogen. The MMP1 antibody was purchased from Millipore.

Immunohistochemistry—Immunohistochemical analysis was carried out for paraffin-embedded, surgically resected specimens of breast cancer. Briefly, the sections were de-paraffinized, rehydrated, and heated at 100 °C for 20 min in Tris-buffered saline (pH 9.0) for antigen exposure. They were treated with 3% H_{2}O_{2} to block endogenous peroxidase activity, blocked by 5% BSA solution for 30 min, and then incubated with primary antibody for 16 h at 4 °C. After washing in PBS with 0.1% Tween 20, the sections were treated with horseradish peroxidase-conjugated rabbit-specific IgG (Dako Corp.). The sections were washed extensively, and 3,3-diaminobenzidine substrate chromogen solution was applied followed by counterstaining with hematoxylin. For negative control, we added IgG instead of primary antibody during the primary antibody incubation. The expression of MMP1 and COX2 were evaluated by immunohistochemistry scoring as the sum of the frequency and the intensity (0, none; 1, weak; 2, moderate; 3, strong). Immunohistochemistry scores were determined by concordance among the scores of two independent reviewers.

Immunocytochemistry—For immunocytochemistry of tight junction proteins, brain endothelial cells were grown to confluence on glass coverslips. The monolayers were treated with the recombinant MMP1- or 231BrM-conditioned medium for 24 h. Cells were washed with PBS and fixed with 3.7% paraformaldehyde. Cells were then permeabilized with 0.1% Triton X-100 in PBS and treated with 1% BSA in PBS followed by incubating with primary antibodies overnight at 4 °C. Primary antibodies were used at the following dilutions: rabbit anti-ZO-1 (1:50), rabbit anti-Occludin (1:50), and rabbit anti-Claudin-5 (1:100). Cells were then washed and further incubated with the appropriate secondary Alexa 546-conjugated goat anti-rabbit antibody (1:200) for 1 h. The cells were fixed onto slides with DAPI and examined under a fluorescent microscope. For examining activated astrocytes, UC-1 cells were grown on coverslips and incubated with Alexa® 488 anti-glial fibrillary acidic protein (1:100) antibody and counterstained with DAPI (1:100) after treatment. To check the CCL7 expression after prostaglandin treatment to astrocytes, CCL7 antibody from RayBiotech was used as primary antibody. The secondary antibody was goat anti-mouse IgG antibody labeled with green fluorescent Alexa Fluor® 488 dye.

Animal Experiments—For the experimental metastasis assay, cancer cells in 100 μl of PBS were injected into the left cardiac ventricle of the nu/nu mice (7–8 weeks). To confirm a successful injection, we immediately monitored photon flux from whole body of the mice. The metastatic growth of tumors in the brain was monitored and quantified by measuring luminescence at brain using Xenogen bioimager. For GM-6001 treatment, the drug was administered intraperitoneally every 3 days (100 mg/kg) and was suspended in PBS with 10% DMSO. The treatment was started 1 day before injection of tumor cells.

Mammosphere Cell Culture—Tumor initiating cells were prepared by the MACS methods as described above, and they were seeded into a 96-well ultra-low binding plate. The cells were incubated in mammosphere culture medium in the presence or absence of conditioned medium for 6 days. The number of spheres per field was measured under a microscope.

ELISA—For assessing the concentration of prostaglandin, conditioned medium from MB231 and 231BrM cells or their tumor initiating cells population were harvested, and they were subjected to the ELISA assay using the PGE_{2} (prostaglandin E_{2})/PGF_{2α} (prostaglandin F_{2α}) assay kit (Cayman Co).

Statistical Tests—For metastasis free survivals, Kaplan-Meier curves were plotted using time to tumor detection as the outcome. Statistical differences in survival across groups were assessed using the log rank test. Cox proportional-hazards regression for multivariate statistical analyses was performed using MedCalc for Windows, Version 14.1 (MedCalc Software, Ostend, Belgium). For analyses of cytokine array result, the expression of cytokines was quantified with ImageJ software. For other analyses, Student’s t tests were used unless otherwise noted. *, **, and *** indicate p < 0.05, p < 0.01, and p < 0.001, respectively. All other statistical analyses were performed using the GraphPad Prism 5 Program (GraphPad Software, San Diego, CA).

RESULTS

Expression of MMP1 Is Positively Correlated to Brain Metastasis of Breast Cancer—To clarify the relevance of all metalloproteinases in brain metastasis, we first analyzed clinical significance of 21 metalloproteinases on brain metastasis-free survival of breast cancer in the cohort of 710 breast cancer patients in the GEO database. As shown in Fig. 1A, we found that only MMP1, -9, and -16 among all metalloproteinases are significantly correlated to brain metastasis in these patients. We then examined the expression of all metalloproteinases in breast cancer cell lines that are known to preferentially metastasize to the brain (MDA-MB231 and CN34BrM) as well as in their parental cells using publicly available databases. Strikingly, among all metalloproteinases, only MMP1 was found to be significantly up-regulated in both brain metastatic cell lines compared with their parental cells (Fig. 1B). This result was also confirmed by qRT-PCR and Western blot analysis using these paired cell lines as shown in Fig. 1C. To further validate the results, we examined the expression of MMP1 in tumors that were metastasized in the brain of breast cancer patients by immunohistochemistry. As shown in Fig. 1D, we found that
MMP1 is indeed strongly expressed in both primary lesions and brain metastatic lesions from breast cancer patients with brain metastasis, whereas primary tumors in patients with high grade but without brain metastasis showed weak expression of this gene. These results strongly suggest that MMP1 plays a pathological role in brain metastasis of breast cancer.

MMP1 Promotes Brain Metastasis by Increasing Permeability of BBB—
To further investigate the roles of MMP1 in brain metastasis, we first used an in vitro model of the blood-brain barrier consisting of brain endothelial cells on the luminal side and astrocytes on the abluminal side of a transwell chamber and examined the transmigrating abilities of brain metastatic breast cancer cells. We found that both 231BrM and CN-BrM cells are significantly more capable of penetrating the layer of brain endothelial cells than their corresponding parental cells (Fig. 2A). On the other hand, 231LM cells, which are derived from the same MDA-MB231 cells and preferentially metastasize to the lung, were not capable of transmigrating through the brain endothelial cells and astrocytes, suggesting that the transmigrating ability of metastatic cells is correlated to the expression of MMP1. To test this possibility, we knocked down the expression of MMP1 in 231BrM cells and examined the transmigrating ability of the cells through brain endothelial cells. As shown in Fig. 2B, knockdown of MMP1 significantly suppressed the ability of transmigration. In addition, the general inhibitor of metalloproteinase, GM6001, also significantly blocked the transmigration ability of 231BrM cell. Furthermore, we found that when the conditioned medium of brain metastatic cells (231BrM and CN34BrM) were added into the transwell assay, the transmigrating ability of their parental cells (MDA231 and CN34) was significantly increased, as shown in Fig. 2C. These results strongly suggest that the secreted MMP1 by 231BrM cell contributes to the ability of the metastatic cells in transmigrating and penetrating the blood-brain barrier. Indeed, when we added recombinant MMP1 in the transwell assay, both MD231 and CN34 showed significantly higher ability of transmigration compared with the control without MMP1 (Fig. 2D). We then measured the ability of metastatic cells to affect the permeability of brain endothelial cell junctions using the same transwell model. As shown in Fig. 2E, we found that the CM of 231BrM significantly decreased TEER, whereas the CM of 231BrM-shMMP1 (MMP1 knocked down cells) and CM treated with GM6001 did not affect the permeability. Consistent with these data, an in vitro permeability assay revealed similar results; CM
of BrM cells significantly increased the permeability of Evans Blue across the monolayer of brain endothelial cells (Fig. 2F), indicating a decreased barrier function of BBB.

Occludin, Claudin, and Zo-1 are known to be the major components of tight junction of BBB (2, 4, 5), and therefore, we examined the possibility that these proteins are targets of MMP1. Human brain endothelial cells were cultured in a monolayer, and they were treated with the conditioned medium of 231BrM cells or recombinant MMP1 followed by assays for Occludin, Claudin, and Zo-1 by Western blot. As shown in Fig. 2G, when the brain endothelial cells were treated with either conditioned medium or recombinant MMP1, the amounts of both Occludin and Claudin-5 were strongly reduced, whereas Zo-1 was not affected by either treatment, suggesting that Occludin and Claudin-5 are degraded by MMP1 secreted by 231BrM cells. To further support this notion, we performed immunocytochemistry for the brain endothelial cells, which were treated in the same manner. We found that the expressions of Occludin and Claudin-5 were strongly reduced after treatment of the cells with either the conditioned medium or recombinant MMP1, the amounts of both Occludin and Claudin-5 were strongly reduced, whereas Zo-1 was not affected by either treatment, and strongly supports the results of the Western blot (Fig.

**FIGURE 2.** MMP1 promotes brain metastasis by degrading blood brain barrier. A, the left figure illustrates the model of trans brain endothelial cell migration assay. Various brain metastatic (231BrM, CN34BrM, 4T1) and lung metastatic (231LN) cell lines and their parental cells (231, CN34) were labeled with GFP, and they were subjected to trans brain endothelial cell migration assay. The number of invaded cells was visually counted under a fluorescent microscope. B, the upper panel shows the results of Western blot for 231BrM cells expressing shRNA to MMP1. The transmigrating abilities of MB231, 231BrM, and 231BrM-shMMP1 as well as for 231BrM cells treated with 25 μg/mL metalloproteinase inhibitor, GM6001 (Millipore), were examined by the trans-endothelial migration assay (lower panel). C, MB231 and CN34 were treated with the CM prepared from 231BrM and CN34BrM, respectively, followed by assaying for the trans-endothelial cell migration ability. D, the effect of 1 μg/ml recombinant MMP1 (Peprotech) on the trans-endothelial cell migration ability of MB231 and CN34 was measured. E, TEER was measured for brain endothelial cells that were treated with the CM of 231BrM or 231BrM-shMMP1. The effect of CM from 231BrM treated with GM6001 was also measured. F, permeability of brain endothelial cells for the same set of CM as described in E was measured by the Evans Blue Albumin (EBA) permeability assay. G, brain endothelial cells were treated with the CM of 231BrM or recombinant MMP1 for 24 h followed by Western blot analysis for Occludin, Claudin-5, and Zo-1. H, in the control group, brain endothelial cells were treated with serum-free medium or CM of 231, whereas CM of 231BrM or recombinant MMP1 were used in the test groups for 24 h, and the cells were immunostained for Occludin, Claudin-5, and Zo-1. *, **, and *** indicate p < 0.05, p < 0.01, and p < 0.001, respectively.
COX2-MMP1 Pathway Promotes Brain Metastasis

FIGURE 3. COX2 promotes brain metastasis by enhancing BBB permeability. A, relationship of the expressions of 17 brain metastasis signature genes with MMP1 was examined using the same cohort database used in Fig. 1A. Out of 17 genes, only 4 showed a significant relationship to MMP1. B, the relationship of the COX2 expression with brain metastasis-free survival of patients was examined by Kaplan-Meier analysis using the same cohort data set used in Fig. 1A. C, COX2 expression was examined for the primary tumors (n = 4) and brain metastatic lesions (n = 7) by qRT-PCR. D, breast tumor tissues from the patients without brain metastasis (n = 12) and brain metastatic tumor tissue (n = 10) from breast cancer patients were compared for COX2 expression by immuno-histochemistry. Scale bar = 50 μm. E, expression of COX2 in the brain metastatic cell lines and their parental cells was examined by qRT-PCR and Western blot. F, secreted PGE2 and PGF2α levels in the conditioned medium of MDA231 and 231BrM were measured by the ELISA kit. G, the ability of trans-endothelial migration of MB231, 231-COX2, 231BrM, and 231BrMshCOX2 was examined. The effect of 3.4 μM COX2 inhibitor, NS398 (Cayman), on 231BrM cells was also tested. H, the effect of conditioned medium of 231BrM cell with or without NS398 on the trans-endothelial migration ability of MB231 cell was examined. J, the effect of conditioned medium of 231BrM cells with or without treatment of NS398 on brain endothelial cells was examined for the TEER. J, the effect of conditioned medium of 231BrM cells with or without the treatment of NS398 on BMEC was examined by the Evans Blue Albumin (EBA) permeability assay. ** and *** indicate p < 0.01 and p < 0.001, respectively.

These results indicate that Occludin and Claudin-5 are the targets of MMP1 and that degradation of these proteins contributes to the increased permeability of BBB.

COX2 is Overexpressed in Brain Metastatic Cells—How MMP1 is up-regulated in brain metastatic cells is an intriguing question. To address this issue, we first examined the correlation of the expression between MMP1 and 11 other genes, that were previously defined as a brain metastatic signature by the Massagué and co-workers (46), using the existing cohort database, which includes 47 samples of breast cancer patients with brain metastasis. We found that four genes (PTGS2, ANGPTL4, PLOD2, and B4GALT6) were significantly correlated to the expression of MMP1 and that COX2 (PTGS2) showed the strongest correlation among these genes (Fig. 3A). Furthermore, when we examined patients’ survival using existing data in the GEO bank, we found that high expression of COX2 was significantly correlated to brain metastasis-free survival (Fig. 3B). These results suggest that COX2 plays an active role in
COX2-MMP1 Pathway Promotes Brain Metastasis

brain metastasis of breast cancer patients. We also examined the expression of COX2 in clinical samples of brain metastatic lesions and primary tumors by qRT-PCR (Fig. 3C) as well as immunohistochemistry (Fig. 3D) and found that COX2 is indeed significantly up-regulated in the brain metastatic sites. To further confirm this result, we measured the expression of COX2 in brain metastatic cell lines (231BrM, CN34BrM) by RT-PCR and Western blot. As shown in Fig. 3E, the expression of COX2 in 231BrM and CN34BrM was indeed significantly up-regulated when compared with their parental cells. Notably, the amount of prostaglandins E2 and F2α, end products of COX2, in the culture medium of metastatic cells was also significantly increased in these cells (Fig. 3F). To further test the functional significance of COX2, we examined the transmigrating ability of MDA231 cells with ectopic expression of COX2 and 231BrM cells expressing shRNA to COX2, using the transwell assay system consisting of brain endothelial cells and astrocytes. As shown in Fig. 3G, the ectopic expression of COX2 significantly increased the transmigrating ability of MDA231, whereas knockdown of COX2 in 231BrM cells significantly decreased the transmigrating ability. The addition of COX2 inhibitor, NS398, in this assay system also significantly reduced the transmigrating ability of 231BrM. Furthermore, although the transmigrating ability of MDA231 was significantly augmented by the addition of conditioned medium of 231BrM as shown in Fig. 2C, this effect was significantly suppressed by adding NS398 in the assay system (Fig. 3H). These results indicate that COX2 and its product, prostaglandin, play a role in BBB penetration of brain metastatic cells. This notion is further supported by our result of the TEER assay and the Evans Blue Albumin permeability assay as shown in Fig. 3, I and J. Collectively, our results strongly suggest that both MMP1 and COX2 are critical factors in the early stage of brain metastasis.

COX2 Up-regulates MMP1 Expression—To further investigate the relationship between COX2 and MMP1 in breast cancer brain metastasis, we divided patients into four groups based on the MMP1 and COX2 expression levels (Fig. 4A). We found that breast cancer patients with high COX2 and high MMP1 have the greatest risk of brain metastasis. Furthermore, the result of multivariate analysis indicates that COX2 and MMP1 have more influence on brain metastasis compared with other organ metastases. The patient group with high COX2 and high MMP1 showed a significantly higher hazard ratio compared with the group with high expression of only COX2 or MMP1, which suggests a strong connection between COX2 and MMP1 in brain metastasis. To examine whether COX2 directly controls the expression of MMP1, we measured the level of MMP1 in 231BrM cells in the presence or absence of NS398. We found that NS398 strongly suppressed the expression of MMP1 as shown in Fig. 4B, and this effect of NS398 was reversed by the addition of PGE2 or PGF2α (Fig. 4C), suggesting that COX2 up-regulates MMP1 through prostaglandin. To further test this possibility, we ectopically expressed COX2 in MDA231 and assayed the expression of MMP1 by qRT-PCR and Western blot. As shown in Fig. 4D, the expression of COX2 significantly up-regulated the expression of MMP1 at both the RNA and protein levels. In addition, we introduced shRNA targeting COX2 in 231BrM cells followed by assaying MMP1 expression by Western blot and qRT-PCR. We found that knockdown of COX2 by continuous expression of sh-COX2 significantly suppressed the expression of MMP1 (Fig. 4E). These results indicate that COX2 is able to up-regulate MMP1 via generation of prostaglandin, which in turn activates the expression of MMP1 in brain metastatic breast cancer cells.

Prostaglandin-activated Astrocytes Promote Self-renewal of Cancer Stem-like Cells—Several lines of evidence indicate that COX2 and prostaglandin are involved in promoting the proliferation of cancer stem-like cells and tumor metastasis, although the exact mechanism is not yet understood (30–32). To elucidate the role of the COX2-prostaglandin pathway in cancer stem-like cells in brain microenvironment, we first isolated tumor initiating cells from MB231 and 231BrM cells using CD24, CD44, and ESA as selection markers as described previously (33) followed by testing their metastatic ability by the limiting dilution analysis in nude mice. As shown in Fig. 5A, the isolated tumor-initiating cells showed significantly stronger ability of tumorigenesis compared with the non-tumor initiating cells. Furthermore, these tumor-initiating cells prepared from 231BrM cells expressed significantly higher levels of MMP1 and COX2 compared with the tumor-initiating cells prepared from MBA231 cells (Fig. 5B). These cells were also capable of producing PGE2 and PGF2α in the cultured medium at a significantly higher level compared with their parental cells (Fig. 5C). Because prostaglandin is known to be involved in neurogenesis and mobilizing astrocytes in the brain, we tested a hypothesis that prostaglandin activates astrocytes and generates a “niche” for the growth of tumor initiating cells. As shown in Fig. 5D, when astrocytes were treated with PGE2 or PGF2α, these cells indeed strongly expressed the glial fibrillary acidic protein, which is a marker for activated astrocytes. We pre-treated astrocytes with PGE2 or PGF2α, collected the conditioned medium that was further added to 231BrM cells followed by measuring the tumor initiating cell population by FACS. As shown in Fig. 5E, we found that pretreatment of astrocytes with prostaglandins significantly augmented the ability of astrocytes cells to promote self-renewal of tumor initiating cells. This result was further confirmed by sphere formation assay in the similar culture system (Fig. 5F). These results strongly suggest that prostaglandin produced by cancer cells activates astrocytes, which in turn promotes the self-renewal of tumor-initiating cells, possibly through secretory growth factor(s) or cytokine(s). To address this question, we performed an antibody-based cytokine array analysis (RayBiotech Co.) using the conditioned medium of astrocytes that was treated with or without prostaglandin. We found that CCL7 is the most significantly up-regulated protein by PGE2 among 120 tested cytokines/growth factors (Fig. 5G), and we confirmed the results by qRT-PCR, Western blot, and immunocytochemistry (Fig. 5H). Furthermore, we found that inhibition or knockdown of CCL7 in astrocytes significantly compromised the effect of prostaglandin to stimulate positive feedback loop from astrocyte to tumor initiating cells (Fig. 5, I and J). These results indicate that CCL7 acts as the key regulator for the interaction between cancer cell and astrocyte. Next, because CCL7 is known to promote proliferation of breast cancer cells (34), we examined whether CCL7 augments the self-
renewal of tumor initiating cells. We found that the expression of Nanog, a key stem cell gene, was significantly up-regulated in 231BrM cells by the CCL7 treatment, as shown in Fig. 5K. We also treated 231BrM cells with CCL7 followed by the FACS incubation, cells were collected, and the cell extracts were subjected to Western blot analysis.

FIGURE 4. COX2 promotes the MMP1 expression in brain metastatic cells. A, patients were divided into four groups: COX2 high/MMP1 high (n = 175), COX2 high/MMP1 low (n = 130), COX2 low/MMP1 high (n = 109), and COX2 low/MMP1 low (n = 146), and brain metastasis-free survival was compared among these four groups. Univariate and multivariate analyses were also done for these four groups for brain lung and bone metastasis (COX2 low/MMP1 low group was used as a control). B, 231BrM cells were cultured in the presence or absence of the COX2 inhibitor, NS398, for 48 h, and the expression of MMP1 was examined by Western blot. C, 231BrM cells were cultured in the presence of NS398 (3.4 μm) with or without PGE2 or PGF2α for a rescue experiment. After 48 h of incubation, cells were collected, and the cell extracts were subjected to Western blot analysis. D, the expression of COX2 and MMP1 was examined for MDA231 and MDA231-COX2 by Western blot (left panel) and qRT-PCR (right panel). E, the expression of COX2 and MMP1 was examined for 231BrM and 231BrM-shCOX2, which expresses shRNA to MMP1, by Western blot (left panel) and qRT-PCR (right panel). ** and *** indicate p < 0.01 and p < 0.001, respectively.

To further validate our result in vivo, we have used our animal models of brain metastasis. We prepared tumor initiating cells from (i) 231BrM, (ii) 231BrM-shMMP1, which expresses shRNA to MMP1, and (iii) 231BrM-shCOX2, which expresses shRNA to COX2. They were implanted into nude mice by intracardiac injection followed by monitoring brain metastasis with IVIS Biomager for 4 weeks. As shown in Fig. 6, A and B, ectopic expression of shRNA to MMP1 or COX2 significantly suppressed the growth of brain metastasis. Furthermore, periodic injections of GM6001, a BBB-permeable metalloproteinase inhibitor, into the mice that received implants of 231BrM cells showed dramatic reduction of brain metastasis (Fig. 6, A and B). On the other hand, when MDA231 cells that ectopically expressed the COX2 gene (MDA231-COX2) were implanted into nude mice through intra-cardiac injection, we found that MDA231-COX2 cells showed significantly higher incidence of brain metastasis (Fig. 6C). These results strongly support our notion that the COX2-MMP1 pathway indeed promotes brain metastasis of breast cancer. Fig. 6D illustrates our current model of the pathological mechanism of brain metastasis.
DISCUSSION

The brain is protected from tumor cell invasion by BBB, and even after successful invasion, the metastatic cells must adapt themselves in the brain microenvironment, which is utterly different from that of the primary site (35, 36). Therefore, the failure of the BBB is considered as the first critical step of brain metastasis. The tight junction of BBB consists of transmembrane molecules, Occludin, Claudin, and junctional adhesion proteins that are linked to the actin cytoskeleton through cytoplasmic accessory proteins including ZO-1, ZO-2, and ZO-3 (5, 6). Occludin and Claudin were identified particularly as critical proteins of BBB integrity (37–41). In this study we have shown
that MMP1 is highly expressed in brain metastatic cells, and it is capable of degrading Claudin and Occludin but not Zo-1. Importantly, our cohort data analysis indicate that MMP1 is significantly correlated to brain metastasis-free survival of breast cancer patients. Indeed, our results of immunohistochemistry analysis of clinical tissues revealed that MMP1 is strongly expressed in brain metastatic lesions. Furthermore, knockdown of MMP1 in brain metastatic cells significantly blocked their ability of brain metastasis in vivo, and the BBB-permeable MMP inhibitor, GM6001, significantly suppressed the brain metastasis. Therefore, MMP1 plays a critical role in brain metastasis of breast cancer, and this protein may serve as a therapeutic target. Although the general inhibitor of MMPs did not show a clinical benefit in the past clinical trials, development of more specific inhibitor to MMP1 and use of such drug for a stratified patient group may become an effective strategy.

The roles of various metalloproteinase in tumor progression and metastasis are well documented in various tumors. Breast cancer cells often express high levels of MMP1, -2, -3, -9, -13, and -14 that are involved in degrading the extracellular matrix (42). Ectopic expression of MMP3 in transgenic mice was reported to develop mammary gland tumors, whereas suppression of MMP3 blocked the invasive ability of breast tumor cells (43). In addition, high levels of MMP2 and -3 are known to be correlated to poor prognosis of breast cancer (44). It should be noted that Massagué and co-workers (12, 13) previously identified MMP1 as lung metastasis signature and showed that MMP1 and -2 promoted lung metastasis by facilitating the assembly of new tumor blood vessels (13). They also proved that MMP1’s role of engaging EGF-like ligands increases the risk of breast cancer bone metastasis (45). Interestingly, MMP1 was also shown to be a part of the brain metastasis signature, which consists of 17 genes, whereas the detailed role involved remains unknown (46). In this report we have shown that MMP1 indeed promotes brain metastasis by increasing the permeability of gap junction of BBB through degradation of Claudin and Occludin. Our result is consistent with the previous observation by Liu et al. (47), who showed that knockdown of MMP1 blocked primary growth and distant metastasis of breast

**FIGURE 6. Knockdown of MMP1 or COX2 gene blocks brain metastasis in vivo.** A, 231BrM, 231BrM-shCO2, and 231BrM-shMMP1 were transplanted into female nude mice (n = 10) via intra-cardiac injection. The group that received 231BrM cell was also treated with the BBB-permeable metalloproteinase inhibitor, GM6001 (100 mg/kg), which was administered to mice every 3 days for 4 weeks. The growth of brain metastasis was monitored by measuring the luciferase activity assayed by IVIS Bioimager. At the end point (day 28), mice were sacrificed, and the luciferase signals in the brain were measured in vivo (right panels). B, the upper panel represents the growth of brain metastasis of mice treated as described in A. The lower panel indicates the results of Kaplan-Meier analysis for brain metastasis-free survival. C, MDA-MB231 and MDA231-COX2 were transplanted into nude mice via intracardiac injection followed by monitoring metastatic growth in the brain by IVIS Bioimager. At the end point (day 40), mice were sacrificed, and the luciferase signal in the brain was measured in vivo (right panel). D, a proposed model for the pathological mechanism of brain metastasis. *** indicate p < 0.001, respectively.
COX2-MMP1 Pathway Promotes Brain Metastasis

cancer cells in an animal model. These results suggest that MMP1 may serve as a potential biomarker for brain metastasis of breast cancer patients. In fact, our cohort data analysis indicates that expression of MMP1 has a significant reverse correlation to brain metastasis-free survival in breast cancer patients.

COX2 has been implicated in the pathogenesis of breast cancer metastasis (48) and was defined as one of the signature genes of lung and brain metastasis (46). We have found that COX2 and its product prostaglandin directly up-regulated the expression of MMP1. Previously, the expression of the COX2 gene was reported to be regulated by MMP1 in macrophage (49), indicating that both genes are reciprocally controlled by a potential feedback mechanism. Many aggressive cancer cells express high levels of COX2, and this gene has been shown to affect BBB permeability (50). In addition, inflammatory conditions are known to be related to MMP expression and metastasis (51, 52). We have shown that prostaglandin is indeed capable of increasing permeability of BBB, and this is partly due to direct up-regulation of MMP1. Importantly, we have shown that a COX2 inhibitor (NS398) effectively blocked both MMP1 expression and BBB permeability, suggesting that a COX2 inhibitor may be used as a prophylactic for preventing brain metastasis.

Tumor microenvironment is considered to play a critical role for the growth of metastatic tumor cells when they establish colonization in the distant metastatic sites (36, 53). Tumor-initiating cells that reach the distant organs need to reprogram stromal cells in the microenvironment to build their niche for proliferation (36). Brain is abundant in astrocytes, and we have previously reported that astrocytes can be activated by metastatic cells to support their growth (33). In this study we have shown that prostaglandin can activate astrocytes to release CCL7, which in turn promotes self-renewal of tumor initiating cells. In this context it is noteworthy that CCL7 (MCP3) functions as a homing factor for myocardial mesenchymal stem cells (54). CCL7 also plays a role in various types of cancers, and this protein was previously found to be a strong chemotactic cytokine for multiple myeloma cells, which likely resulted from a specific homing function of this protein (55). Jung et al. (56) reported that CCL7, which is induced by carcinoma-associated fibroblasts, significantly induced the ability of migration of oral squamous cells. Similarly, CCL7 was found to be positively correlated to invasion and metastasis and patient survival of gastric cancer (57). More recently, Rajaram et al. (34) found that fibroblast-secreted CCL7 is capable of promoting proliferation of breast cancer cells. CCL7 appears to activate G-protein through its receptor and following the MAPK pathway (58), which is known to play a critical role in maintaining stemness of embryonic stem cells (59). Therefore, CCL7 is likely to use the similar pathway to augment the proliferation of tumor initiating cells in the brain, suggesting that CCL7 is a potential therapeutic target. In summary, we have shown that the COX2-PGs-MMP1 axis plays critical role in brain metastasis of breast cancer and that blocking this pathway significantly suppresses brain metastasis (see Fig. 6D). Our results warrant further investigation on this pathway as a novel therapeutic target for brain metastasis possibly by developing more specific drugs to MMP1 and stratifying the target patient population.

Acknowledgment—The Tumor Tissue Core is supported by the Comprehensive Cancer Center of Wake Forest University NCI, National Institutes of Health Grant CCSG P30CA12197.

REFERENCES

1. Cheng, X., and Hung, M. C. (2007) Breast cancer brain metastases. Cancer Metastasis Rev. 26, 635–643.
2. Abbott, N. J., Patabendige, A. A., Dolman, D. E., Yusof, S. R., and Begley, D. J. (2010) Structure and function of the blood-brain barrier. Neurobiol. Dis. 37, 13–25.
3. Abbott, N. J., Rönnbäck, L., and Hansson, E. (2000) Astrocyte-endothelial interactions at the blood-brain barrier. Nat. Rev. Neurosci. 7, 41–53.
4. Dejana, E. (2004) Endothelial cell-cell junctions: happy together. Nat. Rev. Mol. Cell Biol. 5, 261–270.
5. Liebner, S., Kniesel, U., Kalbacher, H., and Wolburg, H. (2000) Correlation of tight junction morphology with the expression of tight junction proteins in blood-brain barrier endothelial cells. Eur. J. Cell Biol. 79, 707–717.
6. Fanning, A. S., Jameson, B. J., Jesaitis, L. A., and Anderson, J. M. (1998) The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton. J. Biol. Chem. 273, 29745–29753.
7. Latorre, I. J., Roh, M. H., Frese, K. K., Weiss, R. S., Margolis, B., and Javier, R. T. (2005) Viral oncoprotein-induced mislocalization of select PDZ proteins disrupts tight junctions and causes polarity defects in epithelial cells. J. Cell Sci. 118, 4283–4293.
8. Fedwick, J. P., Lapointe, T. K., Meddings, J. B., Sherman, P. M., and Buret, A. G. (2005) Helicobacter pylori activates myosin light-chain kinase to disrupt claudin-4 and claudin-5 and increase epithelial permeability. Infect. Immun. 73, 7844–7852.
9. Feng, S., Huang, Y., and Chen, Z. (2011) Does VEGF secreted by leukemic cells increase the permeability of blood-brain barrier by disrupting tight junction proteins in central nervous system leukemia? Med. Hypotheses 76, 618–621.
10. Romero, I. A., Radewicz, K., Jubin, E., Michel, C. C., Greenwood, J., Courraud, P. O., and Adamson, P. (2003) Changes in cytoskeletal and tight junctional proteins correlate with decreased permeability induced by dexamethasone in cultured rat brain endothelial cells. Neurosci. Lett. 344, 112–116.
11. Poola, I., DeWittle, R. L., Marshallleck, J. J., Bhatnagar, R., Abraham, J., and Leffall, L. D. (2005) Identification of MMP-1 as a putative breast cancer predictive marker by global gene expression analysis. Nat. Med. 11, 481–483.
12. Minn, A. J., Gupta, G. P., Siegel, P. M., Bos, P. D., Shu, W., Giri, D. D., Viale, A., Oshen, A. B., Gerald, W. L., and Massague, J. (2005) Genes that mediate breast cancer metastasis to lung. Nature 436, 518–524.
13. Gupta, G. P., Nguyen, D. X., Chiang, A. C., Bos, P. D., Kim, J. Y., Nadal, C., Gomis, R. R., Manova-Todorova, K., and Massague, J. (2007) Mediators of vascular remodelling co-ordinated for sequential steps in lung metastasis. Nature 446, 765–770.
14. Lanningham, F. H., Kun, L. E., Reddick, W. E., Ogg, R. J., Morris, E. B., and Pui, C. H. (2007) Childhood central nervous system leukemia: historical perspectives, current therapy, and acute neurological sequelae. Neuropadiology 49, 873–888.
15. Li, M., Yamamoto, H., Adachi, Y., Maruyama, Y., and Shinomura, Y. (2006) Role of matrix metalloproteinase-7 (matrilysin) in human cancer invasion, apoptosis, growth, and angiogenesis. Exp. Biol. Med. (Maywood) 231, 20–27.
16. Greenwald, R. A. (1999) Thirty-six years in the clinic without an MMP inhibitor. What hath collagenase wrought? Ann. N.Y. Acad. Sci. 878, 413–419.
17. Asahi, M., Wang, X., Mori, T., Sumii, T., Jung, I. C., Moskowitz, M. A., Fini, M. E., and Lo, E. H. (2001) Effects of matrix metalloproteinase-9 gene knock-out on the proteolysis of blood-brain barrier and white matter components after cerebral ischemia. J. Neurosci. 21, 7724–7732.
18. Cunnea, P., McMahon, J., O’Connell, E., Mashayekhi, K., Fitzgerald, U., and McQuaid, S. (2010) Gene expression analysis of the microvascular
COX2-MMP1 Pathway Promotes Brain Metastasis

39. Furuse, M., Hirase, T., Itoh, M., Nagafuchi, A., Yonemura, S., Tsukita, S., and Tsukita, S. (1993) Occludin: a novel integral membrane protein localizing at tight junctions. J. Cell Biol. 123, 1777–1788

40. Ohtsuki, S., Yamaguchi, H., Katsukura, Y., Assashima, T., and Terasaki, T. (2008) mRNA expression levels of tight junction protein genes in mouse brain capillary endothelial cells highly purified by magnetic cell sorting. J. Neurochem. 104, 147–154

41. Wachtel, M., Frei, K., Ehler, E., Fontana, A., Winterhalter, K., and Gloor, S. M. (1999) Occludin proteolysis and increased permeability in endothelial cells through tyrosine phosphatase inhibition. J. Cell Sci. 112, 4347–4356

42. Benson, C. S., Babu, S. D., Radhakrishna, S., Selvarumugan, N., and Ravi Sankar, B. (2013) Expression of matrix metalloproteinases in human breast cancer tissues. Dis. Markers 34, 395–405

43. Sternlicht, M. D., Lohchter, A., Symons, C. I., Huey, B., Rougier, J. P., Gray, J. W., Pinkel, D., Bissell, M. J., and Werb, Z. (1999) The stromal proteinase MMP9/stromelysin-1 promotes mammary carcinogenesis. Cell 98, 137–146

44. Bertucci, F., Nesser, V., Granjeaud, S., Eisinger, F., Adelaïde, J., Tagert, R., Loriod, B., Giaconia, A., Benziane, A., Devillard, E., Jacquemier, J., Viens, P., Nguyen, C., Birnbaum, D., and Hougatte, R. (2002) Gene expression profiles of poor-prognosis primary breast cancer correlate with survival. Hum. Mol. Genet. 11, 863–872

45. Lu, X., Wang, Q., Hu, G., Van Poznak, C., Fleisher, M., Reiss, M., Massagué, J., Kang, Y. (2009) ADAMTS1 and MMP1 proteolytically engage EGF-like ligands in an osteolytic signaling cascade for bone metastasis. Genes Dev. 23, 1882–1894

46. Bos, P. D., Zhang, X. H., Nadal, C., Shu, W., Gomi, R. R., Nguyen, D. X., Minn, A. I., van de Vijver, M. J., Gerald, W. L., Foekens, J. A., and Massagué, J. (2009) Genes that mediate breast cancer metastasis to the brain. Nature 459, 1005–1009

47. Liu, H., Kato, Y., Erzinger, S. A., Kiriakova, G. M., Qian, Y., Palmieri, D., Steeg, P. S., and Price, J. E. (2012) The role of MMP-1 in breast cancer growth and metastasis to the brain in a xenograft model. BMC Cancer 12, 583

48. Heinecz, J. L., Ridnour, L. A., Cheng, R. Y., Switzer, C. H., Lizardo, M. M., Khanna, C., Glynn, S. A., Hussain S. P., Young, H. A., Ambbs, S., Wink, D. A. (2014) Tumor microenvironment-based feed-forward regulation of NOS2 in breast cancer progression. Proc. Natl. Acad. Sci. U.S.A. 111, 6323–6328

49. Reel, B., Sala-Newby, G. B., Huang, W. C., and Newby, A. C. (2011) Diverse patterns of cyclooxygenase-independent metalloproteinase gene regulation in human monocytes. Br. J. Pharmacol. 163, 1679–1690

50. Lee, K. Y., Kim, Y. J., Yoo, H., Lee, S. H., Park, J. B., and Kim, H. J. (2011) Human brain endothelial cell-derived COX-2 facilitates extravasation of breast cancer cells across the brain-blood barrier. Anticancer Res. 31, 4307–4313

51. Ichikawa, Y., Ishikawa, T., Momiyama, N., Kamiyama, M., Sakurada, H., Matsuura, R., Hasegawa, S., Chishima, T., Hamaguchi, Y., Fujii, S., Saito, S., Kubota, K., Hasegawa, S., Ike, H., Oki, S., and Shimada, H. (2006) Matrix-lysin (MMP-7) degrades VE-cadherin and accelerates accumulation of β-catenin in the nucleus of human umbilical vein endothelial cells. Oncol. Rep. 15, 311–315

52. Shuman Moss, L. A., Jensen-Taubman, S., and Stetler-Stevenson, W. G. (2012) Matrix metalloproteinases: changing roles in tumor progression and metastasis. Am. J. Pathol. 181, 1895–1899

53. Nguyen, D. X., Bos, P. D., and Massagué, J. (2009) Metastasis: from dissemination to organ-specific colonization. Nat. Rev. Cancer 9, 274–284

54. Schenck, M. A., Filan, A., Zhang, M., Kiedrowski, M., Popovic, Z., McCarthy, P. M., and Penn, M. S. (2007) Monocyte chemoattractant protein-3 is a myocardial mesenchymal stem cell homing factor. Stem Cells 25, 245–251

55. Vande Broek, I., Assissing, K., Vanderkerken, K., Straetmans, N., Van Camp, B., and Van Riet, I. (2003) Chemokine receptor CCR2 is expressed by human multiple myeloma cells and mediates migration to bone mar-
row stromal cell-produced monocyte chemotactic proteins MCP-1, -2, and -3. *Br. J. Cancer* **88**, 855–862

56. Jung, D. W., Che, Z. M., Kim, J., Kim, K., Kim, K. Y., Williams, D., and Kim, J. (2010) Tumor-stromal crosstalk in invasion of oral squamous cell carcinoma: a pivotal role of CCL7. *Int. J. Cancer* **127**, 332–344

57. Hwang, T. L., Lee, L. Y., Wang, C. C., Liang, Y., Huang, S. F., and Wu, C. M. (2012) CCL7 and CCL21 overexpression in gastric cancer is associated with lymph node metastasis and poor prognosis. *World J. Gastroenterol.* **18**, 1249–1256

58. Ong, V. H., Carulli, M. T., Xu, S., Khan, K., Lindahl, G., Abraham, D. J., and Denton, C. P. (2009) Cross-talk between MCP-3 and TGFβ promotes fibroblast collagen biosynthesis. *Exp. Cell Res.* **315**, 151–161

59. Armstrong, L., Hughes, O., Yung, S., Hyslop, L., Stewart, R., Wappler, I., Peters, H., Walter, T., Stojkovic, P., Evans, J., Stojkovic, M., and Lako, M. (2006) The role of PI3K/AKT, MAPK/ERK and NFκB signalling in the maintenance of human embryonic stem cell pluripotency and viability highlighted by transcriptional profiling and functional analysis. *Hum. Mol. Genet.* **15**, 1894–1913