Human Brain Endothelial Cell-derived COX-2 Facilitates Extravasation of Breast Cancer Cells Across the Blood–brain Barrier

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Abstract. *With improvements in systemic control, metastasis to the brain has been more frequently found in patients with breast cancer. In order to gain access to the brain, breast cancer cells must overcome the blood–brain barrier (BBB), a highly selective filter against cellular and soluble substances. Human brain endothelial cells (HBECs) comprise a major element of the BBB, and breast cancer cells first encounter and pass through them for extravasation. To date, however, the precise role of HBECs in metastasis to the brain is unknown. In this study, we examined how HBECs take part in the extravasation process. In an established in vitro model of the BBB, unexpectedly, the transmigration of breast cancer cells was markedly enhanced in the presence of HBECs than in their absence, suggesting that HBECs facilitate the transmigration of breast cancer cells rather than acting as a barrier against them. We then showed that cyclooxygenase (COX-2) induced from HBECs rather than that from breast cancer cells plays a key role in the transmigration. Moreover, expression of matrix metalloproteinase (MMP-2) mediating the transmigration was induced in HBECs by COX-2 after co-culture with breast cancer cells. Taken together, our results suggest that COX-2 and MMP-2 produced from HBECs facilitate the extravasation of breast cancer cells across the BBB.*

Breast cancer is the most frequent malignant tumor in women. Like other types of cancer, most breast cancer mortality is due to metastasis to distant organs, such as the bone, brain, liver, and lungs (1). With improvements in systemic control, metastasis to the brain has been more frequently found in patients with breast cancer (2). Up to 30% of patients with metastatic breast cancer are estimated to experience metastasis to the brain (3), but thus far, very little is known about the mechanisms of how breast cancer cells metastasize to the brain.

In order to metastasize, cancer cells invade the stroma, and intravasate into the blood and/or lymphatic microvessels. They are then transported, through the circulation, to distant organs, and subsequently extravasate from the lumina of vessels through the vessel wall and form micrometastases in the tissue parenchyma (4, 5).

To extravasate into brain parenchyma, circulating breast cancer cells must pass through the blood–brain barrier (BBB), which limits the passage of cellular and soluble substances from the blood into the brain (2, 3). However, the cellular and molecular mechanisms involved in the transmigration of breast cancer cells across the BBB are poorly understood. Human brain endothelial cells (HBECs) comprise a major element of the BBB, and breast cancer cells must first encounter and pass through them for extravasation (2, 3). To date, the precise role of HBECs in brain metastasis is unknown. In this study, we examined how HBECs take part in the extravasation process.

Materials and Methods

Reagents and cell culture. The human breast cancer cell lines MCF-7 and MDA-MB-231 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). They were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin solution at 37˚C in a 5% CO_2-humidified atmosphere. HBECs were purchased from ScienCell (Carlsbad, CA, USA), and cultured in endothelial cell medium (ECM) supplemented with 5% FBS, endothelial cell growth supplement, and penicillin/streptomycin solution until the seventh passage, according to the manufacturer's instructions.

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Transendothelial migration assay. Migration assays across an HBEC/fibronectin barrier were conducted as previously described (6). Briefly, 2×10^4 HBECs were seeded in Boyden chambers (8 μm pore-size membrane) pre-coated with 0.5 mg/ml of fibronectin (BD Pharmingen, San Jose, CA, USA) 3 days before the migration assay. On the day of the experiment, permeability of the monolayer was confirmed by adding FITC-labeled albumin (Molecular Probes, Eugene, OR, USA) to the upper chamber and monitoring levels of fluorescence in the lower chambers. Chambers that allowed more than 5% of albumin diffusion after 6 hours were discarded. In uncoated chambers, levels of fluorescence equilibrated between compartments within 30 min. To evaluate migration, 2×10^5 breast cancer cells were placed in 100 μl of DMEM with 1% FBS in the upper chambers, and the lower chambers contained 600 μl of DMEM with 10% FBS. After 6 hours at 37˚C, the upper chambers were scraped gently with cotton wool. The migration of cells was observed under a light microscope, and numbers of cells in five random fields were counted at ×40 magnification. For the cyclooxygenase (COX)-2 inhibitor experiments, celecoxib was added to the top compartment of the Boyden chambers 24 hours before the migration assay. Breast cancer cells were trypsinized and resuspended at 2×10^5 in 100 μl of DMEM containing 1% FBS and celecoxib. The cells were added to the upper chambers, and celecoxib was added to the lower chambers containing 600 μl of DMEM with 10% FBS.

siRNA transfection. si-Control, si-COX2, and si-MMP2 were purchased from Dharmacon RNAi Technologies (Rockford, IL, USA). The siRNA transfection was performed using a Basic Nucleofector® Kit for Primary Mammalian Endothelial Cells (Amaxa, Basel, Switzerland), according to the manufacturer's recommendations. Transfected HBECs were seeded in 60-mm dishes, incubated for 24 hours, and then subjected to the transendothelial migration assay.

Gelatin zymography. HBECs were grown onto 12-well plates to 80~90% confluency. Then, HBECs were co-cultured with 3×10^4 breast cancer cells for 24 hours. The enzymatic activity of electrophoretically separated gelatinolytic enzymes in the conditioned media was determined by gelatin zymography, as previously described (7). Zones of gelatinolytic activity were detected as clear bands against a blue background.

Real-time PCR. HBECs and MCF-7 cells were grown onto T175 flasks to 80~90% confluency, respectively. Then, MCF-7 cells were detached and co-cultured with HBECs for 6 hours. Cells were harvested, stained with monoclonal mouse anti-human CD105 MicroBeads (Miltenyi Biotec, Gladbach, Germany), and separated using MACS separators (Miltonyi Biotec, Gladbach, Germany), according to the manufacturer's recommendation. CD105-negative cells were incubated with monoclonal mouse anti-human ErbB-2 MicroBeads (Miltenyi Biotec) and separated according to the manufacturer's instructions. After checking the overall purity of separated cells by fluorescence-activated cell sorting analysis using antibodies against CD105 and ERBB-2, CD105-positive cells (HBECs) and CD105-negative/ERBB-2 positive cells (MCF-7 cells) were subjected to real-time PCR. Real-time PCR was conducted with SYBR Green I (Ambion, Foster City, CA, USA), according to the manufacturer's protocol. The cycling conditions were 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 s and 60˚C for 60 s in an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The results of the real-time PCR data are presented as Ct values, where Ct is defined as the threshold PCR cycle at which the amplified product was first detected. We used the comparative Ct method and compared the RNA expression in samples with that of the control in each experiment.

Luciferase assay. pGL3 vector (0.2 μg; Promega, Madison, WI, USA) containing the *MMP2* promoter region was co-transfected into HBECs with the pRL-SV40 vector (4 ng; Promega) using the Basic Nucleofector® Kit for Primary Mammalian Endothelial Cells (Amaxa), according to the manufacturer's recommendations. Transfected HBECs were grown onto 12-well plates to 80~90% confluency, and then co-cultured with 3×10^4 breast cancer cells for 6 hours. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's protocol.

Statistical analysis of the data. All values are reported as the mean±SEM. Differences were assessed by the two-tailed Student's *t*-test using Excel software. $P<0.05$ was considered statistically significant.

Results

HBECs facilitate the transmigration of breast cancer cells. To study the role of HBECs on the extravasation of breast cancer cells, we used an established *in vitro* model of the BBB, composed of HBEC monolayer cultured on top of a fibronectin-coated Boyden chamber as previously described (6). In this model, HBECs themselves did not transmigrate (data not shown). When breast cancer cells were placed in the upper chambers of the model, unexpectedly, the transmigration of both MCF-7 (estrogen receptor [ER]-positive) and MDA-MB-231 (ER-negative) breast cancer cells increased about threefold on HBEC monolayer as compared to that in the absence of an HBEC monolayer (Figure 1). These results suggest that HBECs facilitate the transmigration of breast cancer cells rather than acting as a barrier against them.

HBEC-derived COX-2 plays an important role in the transmigration of breast cancer cells. Next, we sought to elucidate the molecular mechanism of how HBECs promote this transmigration. Recently, it was reported that tumor cells induce COX-2 expression in human microvascular endothelial cells (8). COX-2 is known to contribute to invasion and metastasis of cancer cells (9-11), and the role of COX-2 in metastasis has been studied in cancer cells (12, 13). To date, however, it has not been determinded whether COX-2 of endothelial cells may play an important role in metastasis. Knockdown of *COX2* in HBECs using *COX2* siRNAs significantly reduced the transmigration of both MCF-7 and MDA-MB-231 cells (Figure 2A). Compared to the siRNA control, the transmigration of MCF-7 and MDA-MB-231 cells was suppressed 83% and 74% by *COX2* siRNAs, respectively. *COX2* siRNA did not affect viability of HBECs nor the

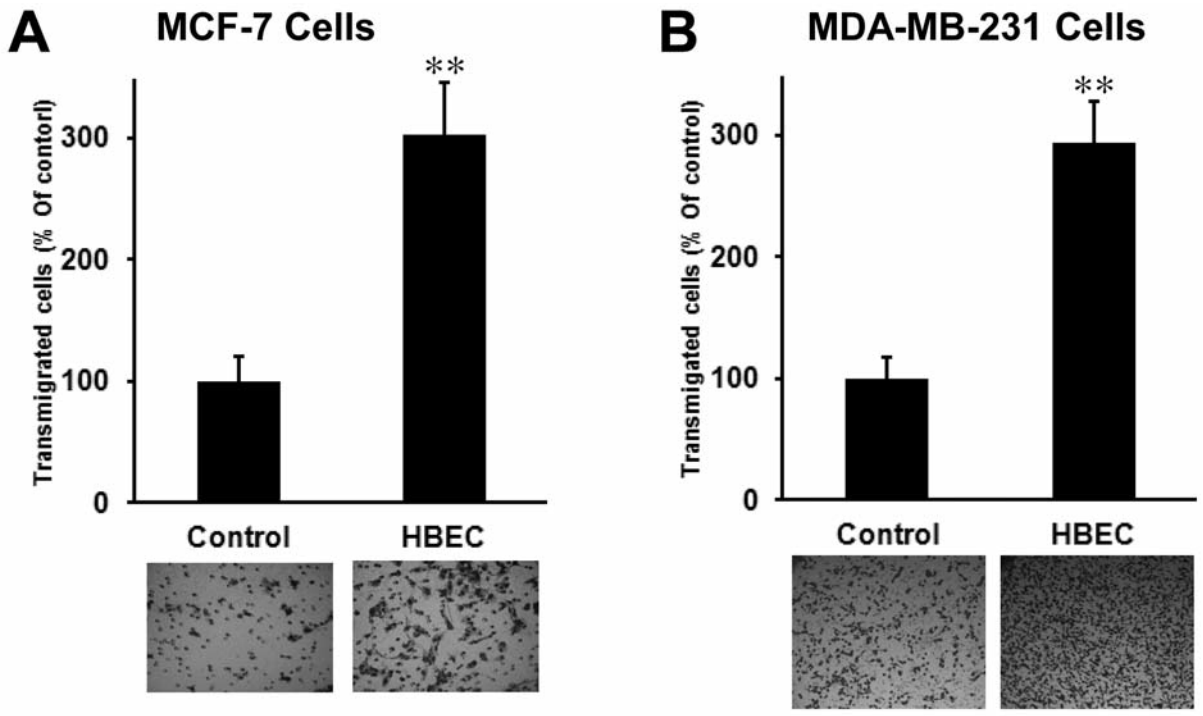

Figure 1. *Differential transmigration of reast cancer cells across the in vitro blood–brain barrier (BBB) model. The relative numbers of transmigrated MCF-7 (A) and MDA-MB-231 (B) cells after 6 hours without (Control) or with HBEC monolayer (HBEC) on the top of fibronectin. **p<0.01 vs. control.*

permeability of the HBEC monolayer (data not shown). Treatment with celecoxib (a COX-2 inhibitor) also suppressed the transmigration of both MCF-7 and MDA-MB-231 cells across the *in vitro* BBB model in a dose-dependent manner (Figure 2B). As compared to the control, 10 μM Celecoxib inhibited the transmigration of MCF-7 and MDA-MB-231 cells by 39% and 41%, respectively.

HBEC-derived MMP-2 is essential for the transmigration of breast cancer cells. It has been reported that COX-2 increases cellular invasiveness through induction of MMP-2 (14, 15). Thus, we tested if MMP-2 mediates the increased transmigration of breast cancer cells by HBEC-derived COX-2. Monocultured HBECs secreted a significant level of latent MMP-2 but not its active form (Figure 3A). In contrast, monocultured breast cancer cells secreted very low levels of latent MMP-2. However, conditioned media from the co-cultures of HBECs and breast cancer cells contained an increased level of both latent and active MMP-2. Celecoxib at 50 μM inhibited the increase in latent and active MMP-2 (Figure 3A). Under all these conditions, MMP-9 was hardly detected.

To clarify the source of the increased MMP-2, HBECs and MCF-7 cells were separated using MACS technology after co-culture and subjected to real-time PCR. *MMP2* mRNA expression in HBECs increased after co-culture with MCF-7 cells (Figure 3B). However, co-culture with HBECs did not change *MMP2* mRNA expression in MCF-7 cells. Thus, the major source of MMP-2 produced by co-culture is from HBECs not MCF-7 cells. We transfected DNA constructs containing an *MMP2* promoter-controlled luciferase gene into HBECs, which were then co-cultured with the breast cancer cells. The luciferase activity was augmented by co-culture with the breast cancer cells (Figure 3C), reconfirming that transcription of the *MMP2* gene in HBECs is induced by co-culture with the breast cancer cells. Moreover, the transfection of *MMP2* siRNA into HBECs reduced the transmigration of breast cancer cells (Figure 3D).

Discussion

The BBB consists of endothelial cells, a basement membrane, pericytes, and astrocytes (16). The endothelial cells are continuously joined by tight junctions and function as highly selective filters against cellular and soluble substances. HBECs cultured on a fibronectin-coated Boyden chamber are widely used as an *in vitro* model system of the BBB, although they lack pericytes and astrocytes (6, 17, 18). This *in vitro* system maintains properties that mimic the *in vivo* BBB. Using this system,

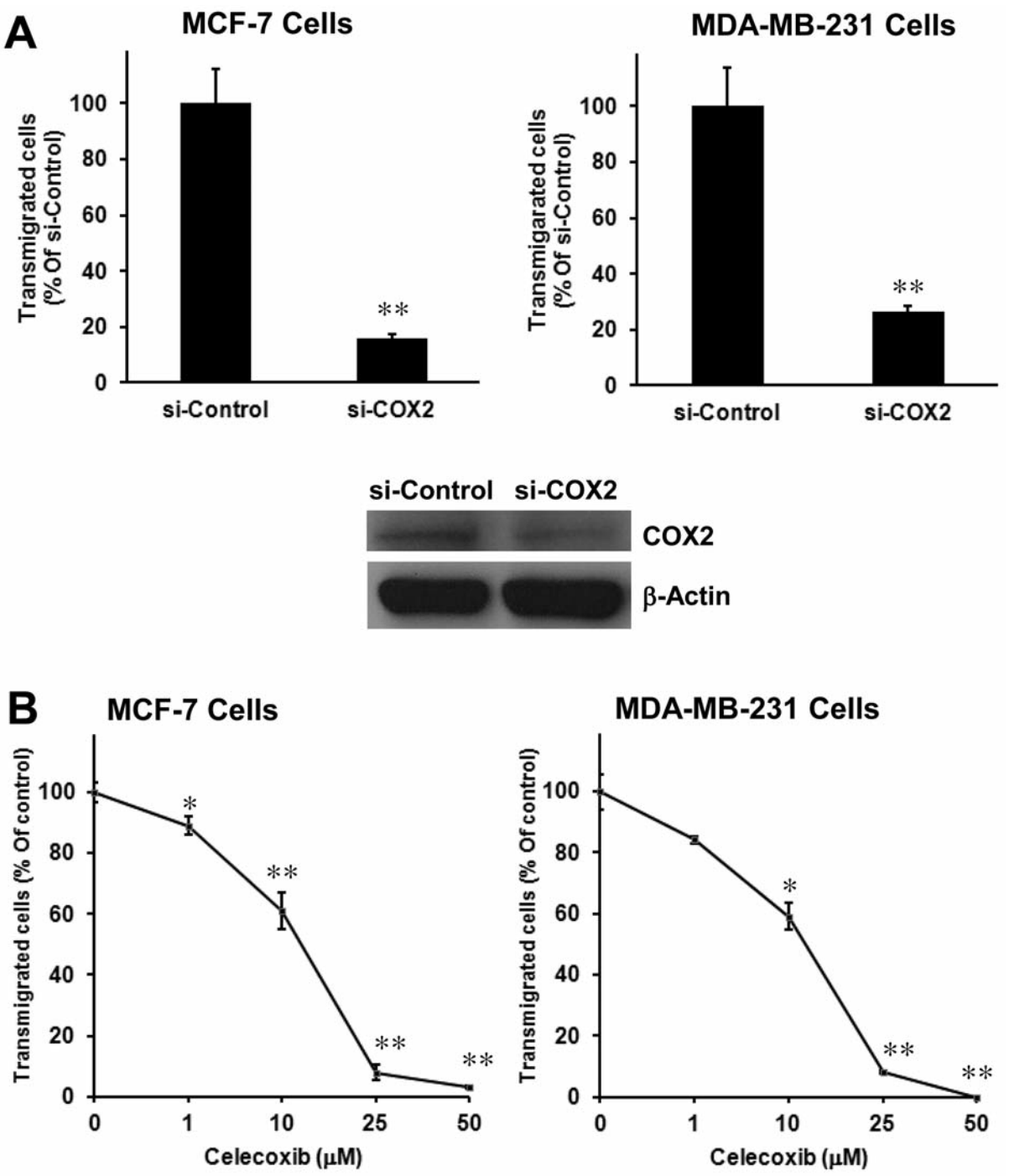

Figure 2. *The role of COX-2 in the transendothelial migration of breast cancer cells. A: The relative numbers of transmigrated MCF-7 and MDA-MB-231 cells after 6 hours across the in vitro BBB model using the HBECs transfected with control siRNA (si-Control) or COX2 siRNA (si-COX2). **p<0.01 vs. control. B: The relative numbers of transmigrated MCF-7 and MDA-MB-231 cells after 6 hours across the in vitro BBB model after treatment with celecoxib. *p<0.05 vs. 0 μM, **p<0.01 vs. 0 μM.*

we showed an active role of HBECs in the extravasation of breast cancer cells across the BBB. HBECs enhanced the transmigration of breast cancer cells. Metastatic breast cancer cells might have just acquired the capacity to overcome the BBB. These results also imply that HBECs dissimilarly respond to different types of cells. Indeed, we previously showed that activated T-cells transmigrate more efficiently across our BBB model than do unstimulated T-

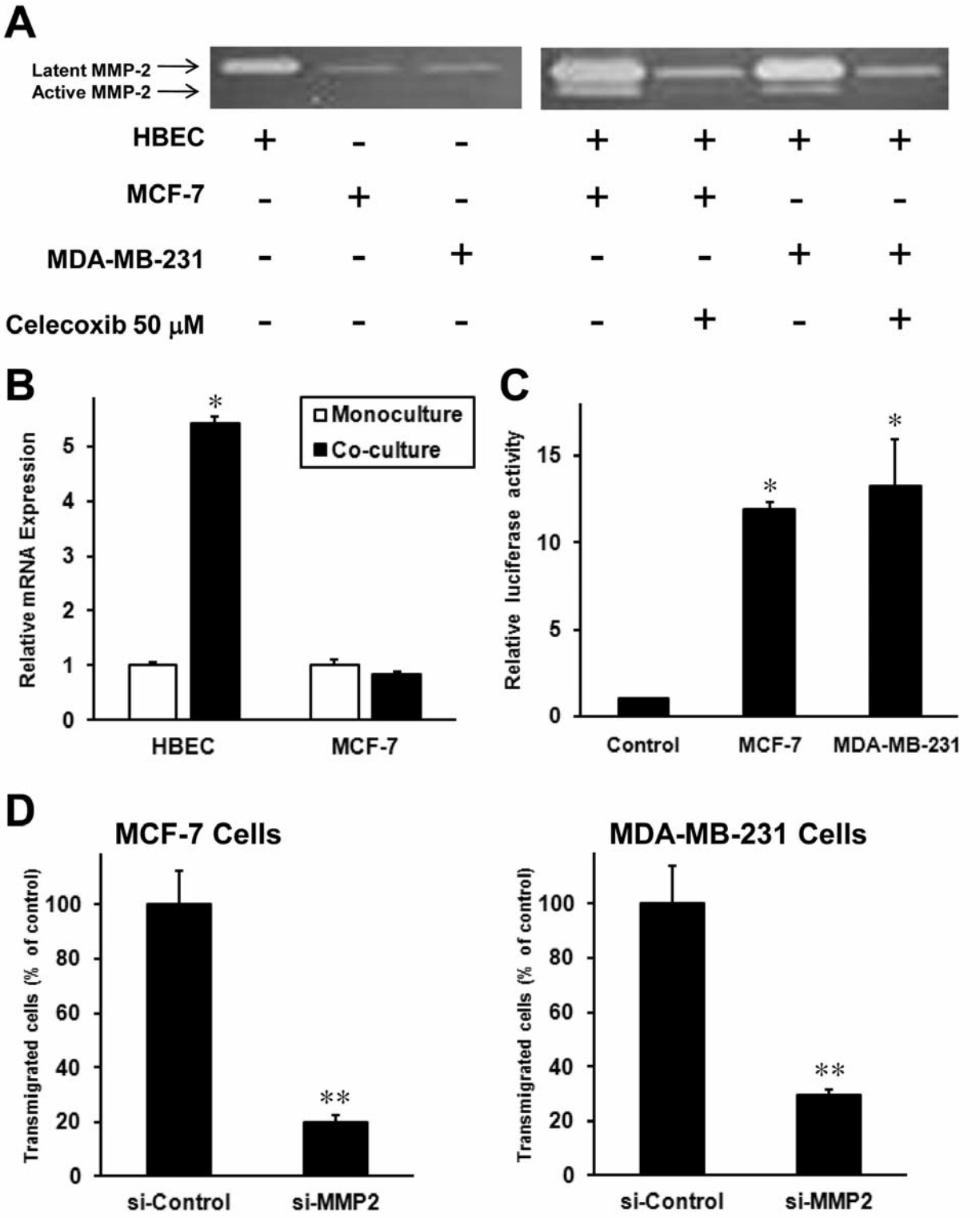

Figure 3. *The role of MMP-2 in the transendothelial migration of breast cancer cells. A: Conditioned media from the monoculture or co-culture of HBECs and breast cancer cells for 24 h were analyzed for MMP-2 activity using gelatin zymography. B: HBECs and MCF-7 cells were separated after co-culture for 6 h, and the separated cells were analyzed for the amount of MMP2 mRNA using real-time PCR. *p<0.05 vs monoculture. C: HBECs were transfected with pGL3 vector containing the MMP2 promoter and cultured without (Control) or with breast cancer cells for 6 h. Then the cells were analyzed for luciferase activity. *p<0.05 vs. control. D: The relative numbers of transmigrated MCF-7 and MDA-MB-231 cells after 6 h across the in vitro BBB model using the HBECs transfected with control siRNA (si-Control) or MMP2 siRNA (si-MMP2). **p<0.01 vs. control.*

cells (6). Thus, a specific interaction between HBECs and breast cancer cells might exist. Gene expression changes in HBECs through interactions with breast cancer cells may be responsible for the enhanced transmigration, being quite distinct from those by T-cells.

COXs are enzymes converting phospholipase A2-mobilized arachidonic acid into lipid signal transduction molecules, prostaglandins and thromboxanes. These products play crucial roles in many physiological and pathophysiological processes, including angiogenesis, development, immunity, and pain (9). COX-1 is constitutively expressed in most normal cells, whereas COX-2 is normally undetectable, but highly inducible at sites of inflammation. Recently, COX-2 has been intensively implicated in tumorigenesis (9-11). COX-2 contributes to various processes, such as activation of carcinogens, tumor initiation and promotion, apoptosis, angiogenesis, invasion and metastasis, and immunological responses. The up-regulation of COX-2 expression occurs in various tumors, such as breast, colon, lung, and gastric cancer, and correlates with poor prognostic parameters, such as distant metastasis and reduced survival. Overexpression of COX-2 has been observed in about 40% of breast tumors (19, 20). Moreover, the importance of COX-2 in the metastasis of breast cancer has been widely studied (21). Most of these studies have mainly focused on the COX-2 expression of breast cancer cells themselves (12, 13). However, our data suggest that COX-2 of HBECs, rather than that of breast cancer cells, plays a predominant role in the extravasation of breast cancer cells across the BBB. The knockdown of *COX2* by siRNA in HBECs markedly suppressed the transmigration of breast cancer cells, although COX-2 from breast cancer cells may also take part in the transmigration.

Celecoxib, a selective COX-2 inhibitor, was originally developed to relieve inflammation and pain while minimizing adverse gastrointestinal side-effects of non-selective non-steroidal anti-inflammatory drugs (22). In this study, celecoxib specifically inhibited the transmigration of breast cancer cells, implying its potential therapeutic value against metastatic breast cancer. Aromatase inhibitors are used for the therapy of hormone-sensitive metastatic breast cancer in postmenopausal patients (23). Recently, a combination therapy of the aromatase inhibitor exemestane and celecoxib has shown synergistic efficacy in such patients (24). Our data may provide a possible explanation for that efficacy.

This study provides supporting evidence that COX-2 induces expression of MMP-2, which is a 72-kDa type IV collagenase, also known as gelatinase A, which degrades components of basement membranes. Pharmacological inhibition of COX-2 by celecoxib suppressed MMP-2 production, which was involved in the transmigration of breast cancer cells. Other studies have also shown that COX-2 promotes MMP-2 expression (14, 15, 25). However, the mechanism is still not well understood. The overexpression of gelatinases (MMP-2 and MMP-9) has been observed in various lines of cancer and is associated with increased tumor invasion and metastasis (26). However, the source of the gelatinase MMPs and their roles in extravasation remain elusive. Endothelial cells constitutively secrete latent MMP-2 under *in vitro* cell culture conditions (27). Similarly, HBECs produced relatively high amounts of latent MMP-2 under our culture system. The secretion of latent MMP-2 was increased by co-culture of HBECs with breast cancer cells, which seemed to be mainly derive from HBECs, because mRNA expression and promoter activity of *MMP2* in HBECs were increased dramatically by this co-culture. In contrast, the basal levels of MMP-2 from breast cancer cells were very low, and real-time PCR analysis showed that *MMP-2* gene expression of MCF-7 cells did not change with co-culture. Thus, it is likely that the main cellular source of MMP-2 is HBECs. Recently, endothelial-derived MMP-2 was described as promoting breast cancer cell transmigration across human lung microvascular endothelial cells (28). Likewise, the inhibition of HBEC-derived *MMP-2* induction by siRNA markedly reduced the transmigration of breast cancer cells, showing that MMP-2 is crucial to the extravasation. These results reconfirm that HBECs play a major role in cancer cell extravasation. Activation of MMP-2 also occurred with the up-regulation of latent MMP-2. This active MMP-2 might be generated by the membrane type 1 matrix metalloproteinase on the surface of breast cancer cells, which initiates a cleavage in the propeptide domain of latent MMP-2 by forming a complex with TIMP-2, a tissue inhibitor of metalloproteinases (26, 27). Indeed, MDA-MB-231 cells were reported to express MT1-MMP (28-30). Accordingly, the specific interaction between HBECs and breast cancer cells may also be important for the activation and expression of MMP-2.

Taken together, our data suggest a crucial role of HBECs in the extravasation of breast cancer cells into the brain. HBEC-derived MMP-2 through COX-2 facilitates the transmigration of breast cancer cells across the BBB. These results reveal an active role of normal cells in the processes of metastasis. To date, most anti-metastasis therapeutic strategies have been developed against cancer cells only. However, this study proposes that the interactions between non-cancer and cancer cells could be potential therapeutic targets against metastasis.

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