Co-culture Microdevice with Oxygen Gradient for Tumor Microenvironment Model and Metastasis Imaging

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Abstract   Tumor hypoxia is a major therapeutic problem since it decreases radiation effects and leads to metastasis. Oxygen is delivered to tumor tissue via abnormal and dysfunctional microvessels, which forms heterogeneity of tissue oxygenation in the tumor. Mimicking the oxygen gradient for cellular experiments *in vitro* is important to clarify the mechanisms involved in tumor biology, but the only method to produce hypoxic conditions at a constant level is using gas-controlled incubators, because there is currently no technique for creating an oxygen gradient using culture dishes. We designed a polydimethylsiloxane (PDMS) microfluidic device integrated with microchannels for cell cultures that enables visualization of cellular distribution under a microscope and co-culture to determine interactions between cancer and other cells. Phosphorescence-based partial oxygen measurements quantified the oxygen gradient, which can be controlled by the gas pressure between the inlet and outlet of the device. A monoculture of endothelial cells with an oxygen gradient in the device showed an increase in cell death in the hypoxic area. In addition, Lewis lung carcinoma cells co-cultured with endothelial cells showed gradient-dependent migration through a membrane pore filter, indicating that the interaction between tumor and endothelial cells under hypoxia is crucial in metastasis. The results suggest that the developed microdevice can be used to study the mechanisms of tumor metastasis under hypoxic conditions.

Keywords   Oxygen Gradient, Microfluidic Device, Co-Culture, Metastasis

1. Introduction

Tumor cells consume oxygen during active division, and abnormal and dysfunctional microvessels form severe hypoxic conditions in the tumors[1, 2]. Tissue hypoxia causes cells to accumulate hypoxia-inducible factor-1 alpha (HIF-1α), which is an oxygen-sensing transcriptional factor that transactivates genes encoding proteins contributing to homeostatic responses to hypoxia[3] and is implicated in tumor metastasis[4]. In addition, hypoxic conditions in tumors decrease radiation effects[5]. To clarify the mechanisms of tumor growth and metastasis and the effects of radiation under hypoxia, cellular experiments in an environment with a controlled oxygen concentration are required.

However, difficulty in reproducing tumor internal environment is a barrier to tumor research *in vitro*. Tumor cells are constantly incubated at 37℃ under 20% O_2 as a control or at 37℃ under 1%–5% O_2 as hypoxia, which does not reproduce the conditions inside tumors. Furthermore, interactions between tumor and other types of cells, such as endothelial cells, pericytes, stromal cells, and immunocompetent cells, are important in tumor metastasis. Co-culture experiments *in vitro* with multiple types of cells are therefore necessary in hypoxia research. Cell culture inserts with a pore membrane are available to culture different cells in wells to examine cellular migration and cell–cell interactions[6, 7]. However, only stepwise changes between normoxia and hypoxia, which do not mimic the physiological oxygen gradient, can be applied to cells.

Recent microfluidic devices for controlling the microenvironment have been used in biomedical research. Microfluidic devices for creating a concentration gradient of a reagent and protein[8] and oxygen gradient[9, 10] have been developed, and various microdevices for cell culture to facilitate microscopic observation of cellular behavior[11, 12] and drug research[13, 14] have been reported; some devices are available for cell cultures with an oxygen gradient[15, 16]. However, the absence of a technique for co-cultures in microdevices to study interactions between cells of different types, has delayed the understanding of mechanisms that relate to tumor metastasis under hypoxic conditions.

In this study, we aimed to develop a co-culture microdevice with an oxygen gradient. To validate our technique, we quantified the oxygen gradient with laser-assisted phosphorimetry and imaged endothelial cells

in the device for viability assay. Finally, we examined the effect of the oxygen gradient on tumor migration by performing co-culture experiments with endothelial cells.

2. Material and Methods

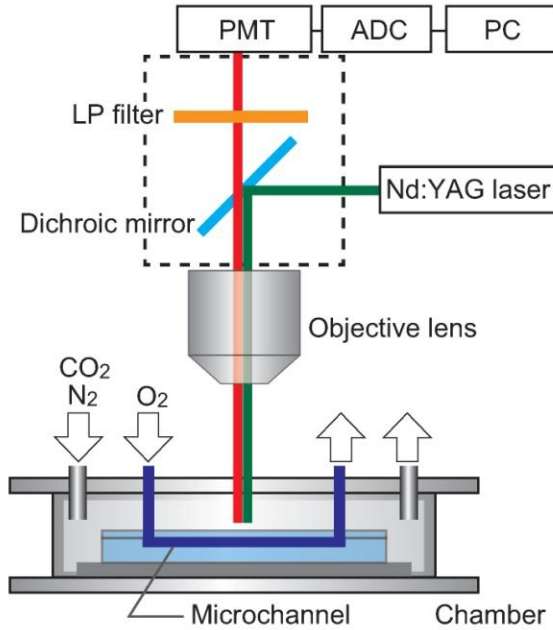## 2.1. Fabricating the co-culture Microdevice

Figure 2. An optical setup for evaluating hypoxic conditions in the microdevice with phosphorimetry. A Nd:YAG laser through an objective lens was irradiated to the PDMS surface exciting Pd-TCPP on it. The emitted phosphorescence depending on oxygen tension was detected with a photomultiplier via a long-pass filter and analyzed to calculate pO_2

The co-culture microdevice, as shown in the cross section in Fig. 1(a), consisted of polydimethylsiloxane (PDMS) with microchannels for gas flow and a membrane pore filter for transmigration of tumor cells, which were located 1 mm above the PDMS coated with collagen for endothelial cell culture. SU-8 (epoxy-based negative photoresist) was spin-coated uniformly on a substrate. A mold with microchannels was designed by photolithography, i.e., by irradiating UV light on the SU-8. Hardener-mixed PDMS was poured into the mold and fixed at 65°C for 1 h. After PDMS was separated from the SU-8 mold, oxygen plasma bonding with a glass substrate was performed. Fig. 1(b) shows the pattern and size of the microchannels, with two microchannels set in parallel and supplying oxygen, such that the central area between the channels became hypoxic. Stainless rings with pore membrane[red area in Fig. 1(b)] were placed on the PDMS substrate for co-culture of tumor cells. The co-culture device was set in a gas-tight chamber made of acrylic plates containing a mixture of 5% CO_2 and 95% N_2, keeping the partial oxygen tension (pO_2) inside the device under 1 mmHg, as shown in Fig. 1(c). The pressure difference between the inlet and outlet for pure oxygen flow

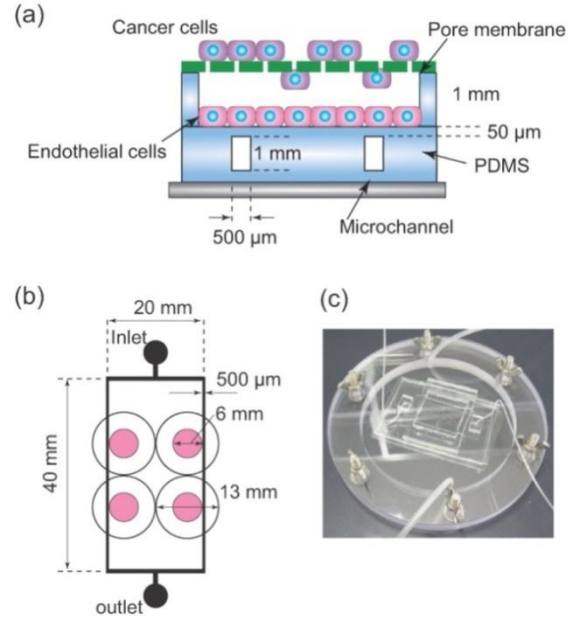was measured by pressure sensors and controlled to change the oxygen gradient in the device.

Figure 1. Schematic diagram of the co-culture microdevice with an oxygen gradient (a). Microfabricated PDMS with microchannels for oxygen supply was attached to a glass substrate through plasma bonding. Tumor and endothelial cells were cultured on PDMS and a membrane filter with 3-μm pore size, respectively. Oxygen gas was supplied from the inlet to the outlet through microchannels, and membrane filters for observation of migrated cells were placed on PDMS (b). The microdevice was installed in a gas-tight chamber filled with a mixture of 5% CO_2 and 95% N_2 (c)

2.2. Measuring oxygen in the cell culture area

Oxygen supplied from microchannels was diffused to the cell culture surface through PDMS. To evaluate the oxygen gradient formed on PDMS, we used laser-assisted phosphorimetry installed on a fluorescence microscope (Fig. 2). pO_2 was measured by oxygen-dependent quenching of phosphorescence, as described previously[17]. In brief, Pd-meso-tetra-(4-carboxyphenyl)-porphyrin (Pd-TCPP, Frontier Scientific, Inc.) was dissolved in phosphate buffer with 5% bovine serum albumin, and the mixture was spread on PDMS. The second harmonic of a Q-switched Nd:YAG pulse laser (532-nm wavelength, 6-ns pulse width at half maximum, 1-Hz pulse recurrence frequency, 200-nJ/pulse irradiation energy) was irradiated at the measurement point through the objective lens of a microscope. Lifetime was obtained by least-squares fitting of the remaining data to a single exponential curve. pO_2 was calculated using the Stern–Volmer equation,

$$\tau_0 \big/ \tau = 1 + k_q \cdot \tau_0 \cdot pO_2 \qquad (1)$$

where I_0 and τ_0 are phosphorescence intensity and lifetime in absence of oxygen, I and τ are phosphorescence intensity and lifetime at a given oxygen tension, and k_q is the rate constant of oxygen quenching. The k_q and τ_0 values for our system were 206 Torr^{-1} s^{-1} and 0.53 ms at 37 °C with a pH of 7.4.

The chamber was filled with nitrogen gas, and the inside pO_2 was maintained under 5 mmHg, creating a hypoxic condition in the device. Pure oxygen gas was supplied from the inlet of the device with pressure differences of 10, 25, 50, 100 mmHg between the inlet and outlet. In addition, pO_2 was measured at points every 1 mm on PDMS for 30 s at a pressure difference of 50 mmHg. A pseudocolor showing pO_2 was imaged using Mathematica 7 (Wolfram Research).

2.3. Cell Culture

Mono and co-cultures can be used in our device. Lewis lung carcinomas (LLC) cells, which have a high metastatic ability, were used to visualize and quantify the distribution of tumor cells during migration, which is dependent on the oxygen gradient. LLCs were grown in RPMI media with 10% FBS, penicillin, and streptomycin. Human umbilical vein endothelial cells (HUVECs) were used for co-culture with LLCs, and were grown in EBM-2 media (Lonza Group Ltd.) with 10% FBS, penicillin, and streptomycin.

To quantify the dependence of cell viability on the oxygen gradient, HUVECs were cultured in a collagen gel, rather than directly on the PDMS surface, so that the cells would adhere to the device surface while alive and could be isolated from the device surface on death. HUVECs were suspended at a cell density of 8×10^5 cells/ml in Cellmatrix (Kurabo Industries Ltd) on ice. Next, 500 μl of the suspension was spread on PDMS with a cell scraper, resulting in a final cell density of 4.4×10^4 cells/cm^2. The device was incubated for 15 min for gelification, and then, 2 ml of EBM-2 was added. The device was further incubated for 12 h under the oxygen gradient.

2.4. Cancer cell migration under the oxygen gradient

Tumor cell migration through the pore membrane under the oxygen gradient, which implied metastasis, was quantified. A part of the pore membrane with the size of 3 μm pore was cut from a 24-well FluoroBlok (BD Falcon) and attached to a circular stainless ring (outside diameter: 16 mm, inside diameter: 6 mm). In the co-culture experiments, HUVECs were cultured on the PDMS surface coated with collagen at a density of 4.4×10^4 cells/cm^2 and LLC cells were seeded at a density of 33×10^4 cells/cm^2, and then incubated separately for 6 h under normoxic conditions. The ring with the membrane was installed on PDMS, and the device was incubated for 12 h under an oxygen gradient formed by the oxygen flow from microchannels. The culture medium for co-culture was a mixture of RPMI and EBM-2 in a ratio of 1:1.

2.5. Fluorescence imaging and processing

Cells were fixed in 4% paraformaldehyde after staining with calcein-AM (Ex: 488 nm; Em: 515 nm) for living cells and propidium iodide (PI, Ex: 530 nm; Em: 615 nm) for dead cells. Fluorescent images were taken using a confocal microscope (TE 2000-U, Nikon), and the number of living and dead cells were counted offline using ImageJ software.

In the experiments on migration, cells that migrated through the pore membrane were fixed and stained with PI, and the average number of cells was calculated from 10 images because the distribution of migrated cells on the membrane was non-uniform.

2.6. Statistical Analysis

Data shown are means $\pm$ SE. The statistical significance of the results was determined using Student's t-test. $P < 0.05$ was considered significant.
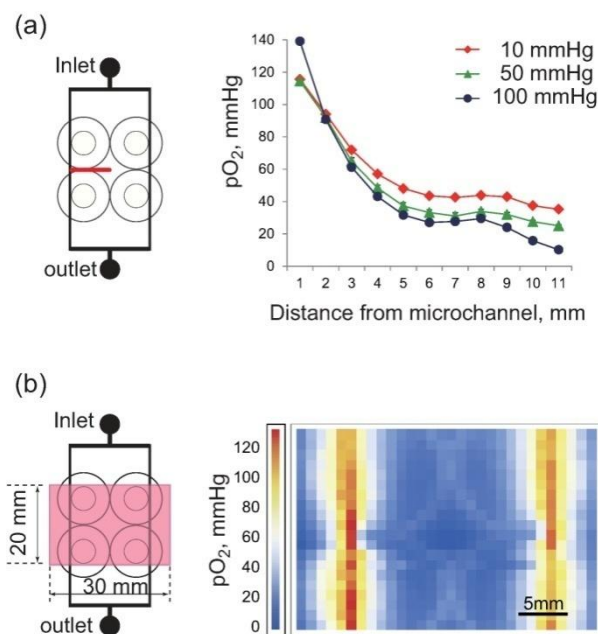

Figure 3. The oxygen gradient in the microdevice, shown by the red line, was quantified when the pressure differences of the pure oxygen gas were 10, 50, 100 mmHg between the inlet and outlet (a). A two-dimensional pO_2 distribution on the PDMS surface was imaged in pseudocolor, indicating oxygen diffusion from the microchannels supplying PDMS with oxygen for cell culture (b)

3. Results and Discussion

3.1. Oxygen gradient in the co-culture microdevice

The only oxygen supply to the co-culture microdevice was via the microchannels in PDMS, since the device was set in a chamber filled with nitrogen gas. Oxygen diffused in PDMS, forming an oxygen gradient with a maximum pO_2 above the microchannels. The oxygen gradient could be controlled by changing the pressure difference of oxygen gas between the inlet and outlet. Figure 3(a) shows the measured oxygen gradient from a microchannel to the central part of the device at pressure differences of 10, 50, and 100 mmHg. pO_2 peaked immediately above the microchannel and decreased exponentially 5 mm away from the channel, and then gently thereafter. The larger the difference in the pressure of the oxygen supply at the inlet and the outlet, the higher the inner pressure of the microchannel, resulting in a higher level of oxygen

diffusion at the inlet than at the outlet; this in turn resulted in a steeper oxygen gradient in the device. Two-dimensional pO_2 distribution on the PDMS surface at a pressure of 50 mmHg is shown in Fig. 3(b). Oxygen was diffused from the microchannels; the lowest pO_2 was at the central part of the device, while the upper part of the microchannels showed no difference between the inlet and outlet.

In the living body, oxygen is delivered mainly by red blood cells and diffused in microcirculation, resulting in an oxygen gradient from microvessels to tissue. In addition, the oxygen gradient depends to a large extent on vessel structure, which is specific to each organ. A major advantage of the microdevice using micro fabrication is that channels can be designed to match an organ-specific vessel structure. For instance, the lobule structure typical of the liver can be designed, producing an organ-specific oxygen gradient.

Different types of theoretical models, for example, Krogh's cylindrical model, have been used to estimate oxygen delivery to tissues. However, theoretical calculations are no match for working with actual tissue; it is extremely difficult to take into account, *in vivo*, the consistency of the simulation and experiments. Cellular experiments *in vitro* play an intermediate role between animal and theoretical experiments. However, most experiments *in vitro* have been performed in standard incubators, indicating that even tumor cells are cultured in about 20% O_2. Most experiments *in vitro* may thus be unrealistic. Most recently, there has been an increase in research on regenerative medicine with ES and iPS cells, and the general consensus is that stem cells should be cultured under hypoxic conditions because a hypoxic micro-environment is critical in stem cell biology. For this reason, a microdevice with an oxygen gradient close to that of physiological tissue holds great potential as a useful tool in various biological experiments.

3.2. Cellular viability under Oxygen Gradient

To validate the oxygen gradient formed in the co-culture microdevice, the viability of the cultured cells was quantified. A mosaic of fluorescent images of cultured HUVECs from the microchannel to 7-mm inside at 50 mmHg of pressure is shown in Fig. 4(a). The green fluorescence of calcein-AM from living cells was dominant on the left side, while the red fluorescence of PI from the nuclei of dead cells increased with distance from the channel, indicating that viability decreased under severe hypoxia. Magnified images located within 1 and 6 mm of the channel in Fig. 4(b) showed that green fluorescence from HUVECs near the microchannels indicated normal cellular shapes, but in the hypoxic area, red fluorescence

increased, and even green fluorescence-emitting cells got rounder, indicating a possible decrease in activity. In Fig. 4(c), plotting the viability of HUVECs with distance from the microchannel, a dramatic decrease in viability was indicated at a distance of 5 mm, with a further significant decrease at 6 or 7 mm. The result of Fig. 3(b) indicates that superficial pO_2 on PDMS at 6 mm from the microchannel was about 30 mmHg. However, HUVECs were inside the collagen gel, and therefore, actual pO_2 around the cells was assumed to be lower than 30 mmHg. These experiments demonstrated that the oxygen gradient in the microdevice affects cellular activity and that severe hypoxia cause death of endothelial cells.
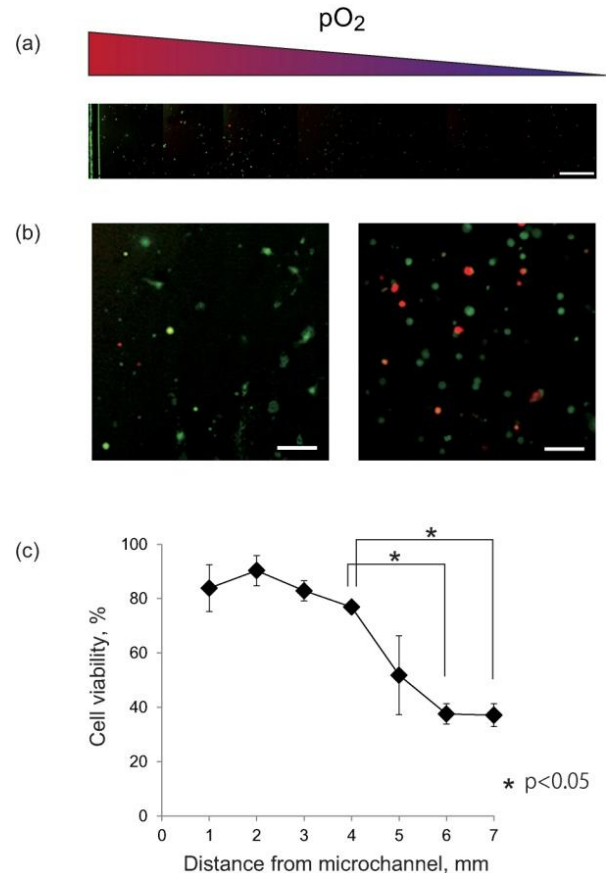

Figure 4.   Cell viability of endothelial cells cultured in collagen gel under the oxygen gradient was evaluated from fluorescence images. Live and dead cells were stained with calcein-AM (green) and PI (red), respectively. A mosaic image from the microchannel to the center of the microdevice showed that viability decreased with distance and oxygen gradient (a, scale bar = 500 μm). In a magnified image near the microchannel, the presence of live cells forming normal shapes was major. However, at 6 mm away from the channel, dead cells increased, and live cells with green fluorescence showed rounded shapes (b, scale bar = 100 μm). Cell viability decreased in accordance with the oxygen gradient in the microdevice (c)

3.3. Metastasis Imaging of Cancer Cells Under An Oxygen Gradient
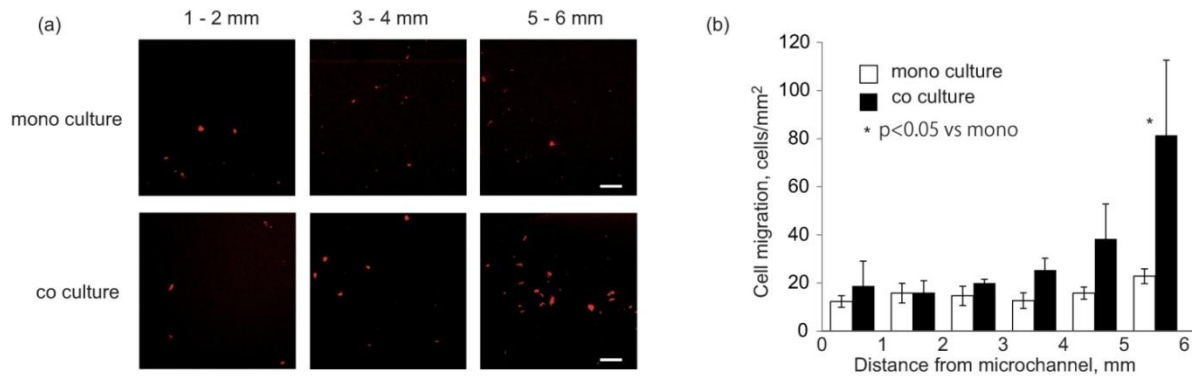
Figure 5. Tumor cell migration mimicking metastasis was observed in the microdevice. LLCs were cultured alone or with HUVECs under an oxygen gradient, and LLCs that migrated through the membrane pore filter were stained with PI (a). Cell migration was quantified using the distance from the microchannel; the hypoxic area with co-culture conditions accelerated the migration of tumor cells (b)

We evaluated cell migration in a culture with endothelial cells under an oxygen gradient to simulate the interior of a tumor. Nuclear staining of LLCs that had migrated to the bottom of the pore membrane was imaged with a confocal microscope, as shown in Fig. 5(a), and cell number/unit area with distance from the microchannel was counted [Fig. 5(b)]. The migrated cells in the monoculture showed minimal increase in the hypoxic area, and in contrast, the co-cultured LLCs with HUVECs increased their migration with distance, i.e., the degree of hypoxia, and from a point at a distance of 4 mm from the microchannel particularly, migrated cells increased notably.

A defining characteristic of the microdevice is that it facilitates the co-culture of tumor cells with other cells under an oxygen gradient. The interaction between tumor cells and endothelial cells, pericytes, or stromal cells is very important *in vivo*, and co-culture experiments have revealed various cellular mechanisms. Fig. 5(b) clearly indicates that not only hypoxic conditions but also the co-culture of tumor cells with HUVECs increased their migration. This confirmed the importance of the microdevice in research applications requiring co-culture under an oxygen gradient.

The device developed in this study has a role in the identification of gene expression in cells and cytokines associated with hypoxia by analyzing cultured cells and culture medium. For instance, HIF-1α is a type of "master switch" of the transcriptional response to hypoxia and is known to be associated with tumor metastasis. Previous studies have provided evidence that Snail and Twist are downstream targets of HIF-1α, and they repress E-cadherin expression in tumor cells that is a major component of adherens junctions [18, 19]. This leads to epithelial–mesenchymal transition in tumor cells, causing metastasis. In endothelial cells, cytokines activating cellular motility are produced in the hypoxic area. MMPs, CXCR4, HGF, and AMF are factors associated with tumor metastasis and invasion. Hypoxia stimulates CXCR4 expression in endothelial and tumor cells [20], and CXCR4 overexpression increases tumor invasion [21]. Our results, as shown in Fig. 5(b), lead to the presumption that co-culture under an oxygen gradient produced a synergistic effect on tumor cell migration through the expression of transcriptional factors and cytokines in LLCs and HUVECs. The addition of an oxygen gradient to co-culture experiments on tumor and endothelial cells has the potential to clarify the details of molecular mechanisms involved in tumor metastasis. Moreover, changing the combination of tumor cells with various other cells enables further discussion in tumor biology, and we can use the device for the evaluation of radiation effects under hypoxia by setting the chamber, including the microdevice, in a radiotherapy unit.

4. Conclusions

We developed a co-culture microdevice with an oxygen gradient to visualize cell distribution and quantify cell viability, and used the device to determine the effect of hypoxia on tumor metastasis. LLC cells co-cultured with endothelial cells showed gradient-dependent migration through a membrane pore filter, indicating that the interaction between tumor and endothelial cells under hypoxia is crucial in metastasis. The results suggest that the developed microdevice can be used to study the mechanisms of tumor metastasis under hypoxic conditions.

ACKNOWLEDGEMENTS

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