A microfluidic platform for quantitative analysis of cancer angiogenesis and intravasation^{a)}

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Understanding the mechanism behind cancer metastasis is a major challenge in cancer biology. Several *in vitro* models have been developed to mimic a cancer microenvironment by engineering cancer–endothelial cell (EC) and cancer–stromal cell interactions. It has been challenging to realistically mimic angiogenesis, intravasation, and extravasation using macro-scale approaches but recent progress in microfluidics technology has begun to yield promising results. We present a metastasis chip that produce microvessels, where EC and stromal cells can be patterned in close proximity to tumor cells. The vessels are formed following a natural morphogenic process and have smooth boundaries with proper cell–cell junctions. The engineered microvessels are perfusable and have well-defined openings toward inlet and outlet channels. The ability to introduce cancer cells into different locations bordering to the microvessel wall allowed generation and maintenance of appropriate spatial gradients of growth factors and attractants. Cancer angiogenesis and its inhibition by anti-vascular endothelial growth factor (bevacizumab) treatment were successfully reproduced in the metastasis chip. Cancer intravasation and its modulation by treatment of tumor necrosis factor- α were also modeled. Compared to other models, the unique design of the metastasis chip that engineers a clear EC–cancer interface allows precise imaging and quantification of angiogenic response as well as tumor cell trans-endothelial migration. The metastasis chip presented here has potential applications in the investigation of fundamental cancer biology as well as in drug screening. © 2014 AIP Publishing LLC.

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INTRODUCTION

Cancer cells constantly interact with surrounding blood vessels during different stages of metastasis.^{1,2} Cancer angiogenesis happens when the colonies grow and begin to secrete factors that stimulate surrounding blood vessels to grow towards the colony. The new blood vessels supply increasing amount of nutrients and oxygen to growing tumors which further recruit more vessels. Cancer cells from colonies penetrate surrounding tissue and blood vessels, circulate in the blood, and enter secondary sites in the perivascular region³ then grow to establish secondary colonies. Therefore, angiogenesis and transendothelial migration have been investigated extensively as they are the main steps for the progression of cancer metastasis. However, it has been difficult to realistically mimic the multiple steps of cancer metastasis *in vitro* due to difficulty in establishing physiological blood vessel models and spatially well-defined

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co-cultures of cancer and endothelial cells (ECs). Therefore, an *in vitro* model that can mimic the metastatic cascade in a well-controlled microenvironment with more realistic blood vessel model is needed for further understanding metastasis.

In transwell assay, cancer cells are grown in the lower chamber, and the upper chamber is cultured with endothelial cells in an extracellular matrix like collagen or Matrigel. Tube formation⁴ or migration of the endothelial cells⁵ can be observed in response to the cancer-derived factors. For modeling the transendothelial migration of cancer cells, endothelial cells are cultured to confluence on a Petri dish or porous membrane, and the penetration of the cancer cells through the endothelial monolayer can be observed following several hours to days of incubation.^{6–11}

Advances in microfabrication technology have facilitated development of *in vitro* models for cancer biology research. Microfluidic technology has several advantages over conventional methods in terms of precise control of microenvironmental factors, and high-resolution imaging of cell motility or cell-to-cell interactions.^{12,13} Several studies have utilized microfluidic technology to model different stages of the cancer metastasis. Kim *et al.* have reported a cancer angiogenesis model by attaching endothelial cells on the sidewall of fibrin gel, and stimulating them by co-culturing with spatially separated cancer cells.¹⁴ A limitation of this system was that the sprouting was originated from endothelial cell clusters without cell-cell junctions rather than preexisting vessels. This is slightly different from *in vivo* cases where cancer angiogenesis sprouts originate from the fully established blood vessels near cancer colonies.

In other microfluidics-based studies, blood vessel wall was modeled by culturing human umbilical vein endothelial cells (HUVECs) as a monolayer.^{15,16} Transendothelial migration was modeled by introducing cancer cells into the system. However, this model of blood vessels involved attaching the endothelial monolayer to a hydrogel without angiogenic or vasculogenic process, which generates the blood vessel network in a living system. Using a similar model described by Kim *et al.*,¹⁴ cancer cell extravasation was reproduced from a blood vessel network that was generated by vasculogenic process.¹⁷ However, this model does not allow directionality of a chemokine gradient for inducing chemotactic migration of the cancer cells, and unpredictable geometrical structure of the blood vessel network increases the complexity of the assay. Therefore, we speculated that overcoming these problems by recruiting vasculogenic process with more predictable geometrical structure of the blood vessels would yield an *in vitro* cancer-EC model that closely mimics the *in vivo* microenvironment.

This paper describes a new metastasis chip design where cancer angiogenesis and transendothelial migration can be assayed in a quantitative and reproducible manner. A robust assay platform was established by forming a microvessel by vasculogenic process with spatially well-defined co-culture tumor microenvironment. The smooth boundary of the microvessel represents a close *in vivo* mimic of pre-existing blood vessel wall. Design of the chip has spatial co-culture flexibility that allows lung fibroblasts (LFs) to stimulate endothelial cells to become a differentiated microvessel, followed by the cancer cell introduction at the perivascular microchannel to model the cancer-EC interactions. The endothelial cells showed an *in vivo*-like elongated morphology with proper cell-cell junctions, and the three-dimensional vascular structure opened to the media channel to allow perfusion of media into the vessel lumen. Cancer angiogenesis and blocking of the angiogenic pathway by treatment with anti-vascular endothelial growth factor (anti-VEGF; bevacizumab) was modeled successfully. Intravasation of cancer and its modulation by tumor necrosis factor- α (TNF- α) treatment was also investigated.

MATERIALS AND METHODS

Cell culture, immunostaining, and reagents

HUVECs (Lonza) were cultured in Endothelial Growth Medium (EGM-2, Lonza). Normal human LFs (Lonza) were cultured in Fibroblast Growth Medium (FGM-2, Lonza). Human glioblastoma multiform cells, U87MG (ATCC, Virginia) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 U/ml). The MDA-MB-231 cells were obtained from ATCC (Manassas, VA). MDA-MB-231 cells were transfected with pEGFP plasmid, followed by selection of the cells

with 1 mg/ml G418 (A.G. Scientific, Inc.). The MDA-MB-231 GFP cells obtained from the single clone were maintained in RPMI 1640 (WELGENE, Korea), supplemented with 10% FBS, 1% penicillin/streptomycin (Gibco, BRL), and 250 μ g/ml G418 (A.G. Scientific, Inc.). All cells were cultured in a humidified incubator at 37 °C and 5% CO₂.

For immunostaining, mouse monoclonal antibody specific for human ZO-1 (Alexa Fluor1594, clone ZO1-1A12, molecular probes), CD31 (AlexaFluor1647, clone WM59, BioLegends), VE-cadherin (eBioscience) and claudin-5 (Invitrogen) were used for endothelial imaging and Hoechst 33342 (molecular Probes) was used to stain the nuclei.

Bevacizumab (Avastin, Genentech) was diluted into 500 μ g/ml with EGM-2 and treated to the microvessels with the cancer introduction in the cancer angiogenesis experiments. For TNF- α treatment, recombinant Human TNF- α (PeproTech) was diluted into 5 ng/ml with EGM-2 and treated into the microvessels starting at 24 h before the cancer introduction.

Microfluidic device fabrication

Microfluidic devices were fabricated with the same protocol described previously.¹⁴ In brief, master mold of the device was fabricated by casting photo resist on a silicon wafer. Standard photo lithography protocol for SU-8 100 (Microchem, US) photoresist was used to make 150 μ m thickness molds. PDMS (Dow Corning, US) was poured on the completed master mold and cured in an 80 °C dry oven for 40 min. The PDMS and cleaned coverslip were bonded together by using plasma treatment (Femto Science, KR). For hydrophobicity, the bonded devices were kept in dry oven with 80 °C for at least 48 h. Entire schematic drawing and detailed dimensions of the chip is shown in supplementary Figure 1.³⁶

FIG. 1. Top-down view of the metastasis chip design scheme and experimental procedures. (a) Schematic drawing of the metastasis chip. The chip consists of seven microchannels, separated by the array of microposts. LFs or HUVECs mixed with fibrinogen solution are loaded into LF and vessel channels, followed by the media injection. (b) Microvessel formation. Leaving bridge and upper channel without any fluid injection, the chip is incubated for 7–8 days for microvessel formation with three-dimensional structure. (c) For cancer angiogenesis assay, U87MGs mixed with fibrinogen solution is injected into the upper channel and fibrinogen solution is injected into the bridge channel. Stimulated by the secretion of U87MG, angiogenic sprouts stemmed from the microvessel wall grow toward the upper channel. (d) For cancer intravasation assay, MDA-MB-231 cells are loaded into the bridge channel and tilted for their attachment on the fibrin wall. Media is filled after the attachment, and followed by several days of incubation, the MDA-MB-231 cells invade into the fibrin gel and intravasate through the microvessel wall.

Hydrogel and cell loading into the metastasis chip

HUVECs ($6 \times 10^6/\text{ml}$ in most experiments, some experiments used 3 or $9 \times 10^6/\text{ml}$ as noted) and LFs ($7 \times 10^6/\text{ml}$) were mixed with a fibrinogen solution (2.5 mg/ml fibrinogen with 0.15 U/ml aprotinin and 0.5 U/ml thrombin) and injected into vessel channel and LF channels, respectively (Fig. 1(a)). After incubating for 2 min to allow for fibrin polymerization, we filled EGM-2 media into the media channel. The devices were incubated for 7–8 days to engineer fully lumenized microvessel with open ends at the each media channel (Fig. 1(b)).

For cancer angiogenesis, U87MGs with fibrinogen solution were injected into the upper channel and bridge channel was filled with fibrinogen solution. For cancer intravasation, MDA-MB-231 cells ($1 \times 10^6/\text{ml}$, ~ 180 cells in one chip) were injected into the bridge channel and tilted for 40 min to attach to the fibrin wall between the bridge and vessel channel. For inducing chemotactic migration of the cancer cells, EBM-2 (media without additional growth factor supplements) was filled into the bridge and upper channel, while EGM-2 media was filled at the media reservoirs.

Microscopy

A confocal microscope (Olympus FV1000) was used for 3D z-stack and cross-sectional imaging. The confocal images were analyzed by IMARIS (Bitplane, Switzerland). IX81 inverted microscope (Olympus) was used for fluorescence imaging.

Data analysis

3D z-stack images were analyzed with IMARIS software. For quantifying the success rate of smooth and continuous boundary of the microvessels, the length of the vessel boundary was measured and compared with the straight-line length of the vessel channel along the microvessel using Image J. We regarded the chip as success if the boundary length value is in a range of $\pm 10\%$ to the straight-line length of the vessel channel. Every microvessels that have disconnected vessel wall was considered as failure.

Permeability coefficient calculation was performed using the same method that was presented previously.¹⁸ In brief, FITC-dextran solution was introduced into the microvessel and fluorescently imaged in every 15 s using multi-stage time-lapse mode in Metamorph. The acquired time-lapse image was analyzed with Image J and permeability coefficient was calculated based on the equation as follows:

$$P = \frac{1}{l_w} \times \frac{dI/dt}{I_i},$$

where l_w is length of the vessel wall that separates between perivascular and microvessel region, I_i is the area averaged intensity in the microvessel region, and I is the total intensity in the perivascular region.

For the quantification of cancer angiogenesis, each two images, acquired from the same sample before and after the cancer or bevacizumab introduction, were compared and the number of additional sprouts or sprout area were manually counted or drawn by using Image J. For quantification of cancer intravasation, the microvessels were stained with CD31 and fluorescence imaged, and cancer cells at the apical side of the microvessel were manually counted using the IMARIS software.

RESULTS

Metastasis chip design

We have previously described formation of a perfusable microvessel network in a microfluidic device using a co-culture system of HUVEC and LF.^{14,19} The HUVEC sprouts were stimulated by LF, which opened their lumens to both sides of the channel, allowing fluid

passage through the vessels. However, the structure of microvessel network was unpredictable, and other cell types could not be introduced into the perivascular region, as the perivascular regions were filled with gel or PDMS wall. Therefore, we generated a microvessel with more predictable geometric characteristics and with perivascular regions that could be filled with other cell types after the generation of the microvessels with vasculogenic process.

Fig. 1 describes the schematic of the metastasis chip and the experimental procedures. Previous design¹⁴ produced microvessels that randomly traverse across the vessel channel. Metastasis chip was designed to establish a single microvessel with well-defined vessel wall for quantitative analysis of the cancer-EC interactions, angiogenesis, and trans-endothelial migration. The chip consists of seven microchannels separated by an array of microposts following a previously described design feature.²⁰ The channels can be categorized into two groups: (1) a vessel channel, two media channels, and two LF channels for microvessel formation, (2) bridge channel and upper channel for cancer introduction (Fig. 1(a)).

We used the same scheme with the previous design for generating a perfusable microvessel, which uses LF channels and a vessel channel connected by media channels to induce a paracrine interaction between LF and HUVEC. In this work, however, instead of positioning LF channels at the both side of the vessel channel as the previous design,¹⁴ the position of LF channels and two openings in the vessel channel is on the same side (lower side). This generates microvessel with two openings on the same side, while the other side of the microvessel is interfaced with another microchannel, which is called the “bridge channel” in Fig. 1(a).

The upper and bridge channels are empty during the microvessel growth. The interface with the empty channel prevents HUVECs in the upper region of the vessel channel from migrating or generating sprouts toward the upper direction of the vessel channel, and further allows the generation of a smooth vessel wall, parallel to the interface. Cancer angiogenesis and intravasation assay could be quantitatively performed by introducing cancer cells into the empty channels.

Figs. 1(b)–1(d) show a schematic drawing of the generation of a microvessel, the performance of the cancer angiogenesis, and intravasation assays using the chip. We first injected an LF-fibrin mixture into the LF channels, and a HUVEC-fibrin mixture into the vessel channel to engineer the microvessel. Media was added to connect the cell-loaded channels, and a microvessel with two openings toward the media channels was generated after a 7–8-day incubation (Fig. 1(b)). Two assays were performed following the generation of the microvessel. We modeled cancer angiogenesis using the U87MG cell line, which is known for its considerable angiogenic potential^{21,22} as a cancer sprout inducer from the microvessel. We injected an U87MG-fibrin mixture into the upper channel, followed by a fibrin injection into the bridge channel (Fig. 1(c)). We observed the formation of cancer sprouts from the pre-existing microvessels toward the cancer site, which was promoted by the secretion of pro-angiogenic factors from U87MG cells. We attached MDA-MB-231 cells to the fibrin gel that was exposed to the bridge channel and supplied media to the upper channel for the cancer intravasation assay (Fig. 1(d)). By supplying growth factor-free media (EBM-2) at the upper channel, we generated increasing gradient of growth factor concentration toward the vessel channel. Stimulated by the growth factor gradient, the cancer cells migrated to the microvessel site at the vessel channel. After a 2–3-day incubation, intravasated cancer cells were observed inside the microvessels, which indicated successful modeling of the cancer intravasation process.

Formation of perfusable microvessels with smooth and continuous boundaries

Engineering smooth and continuous vessel boundaries is necessary for quantitative analysis of angiogenesis and trans-endothelial migration. We left the bridge channel empty to minimize the generation of sprout toward the upper region of the vessel channel during the microvessel formation. Furthermore, HUVEC cell concentration has to be optimized to engineer microvessels with smooth and continuous boundaries. We tested three conditions (3 , 6 , and 9×10^6 HUVECs/ml and 7×10^6 LF/ml) using 4–5 chips per condition, and results are expressed as the mean of three independent experiments. Fig. 2(a) shows the microvessel morphology on day 7

FIG. 2. HUVEC concentration optimization for generating smooth and continuous vessel boundary. (a) Micrograph of the microvessels according to the initial HUVEC concentration at day 7. HUVEC concentration 6×10^6 /ml shows the smoothest and continuous microvessel wall. (b) Success rate of the smooth microvessel wall formation. HUVEC concentration 6×10^6 /ml shows the highest success rate of the smooth vessel wall formation. (c) Time-lapse micrograph of the microvessel formation in 6×10^6 /ml HUVEC concentration. Endothelial cells scattered in the vessel channel fuse together and become a microvessel with patent, perfusable lumen. Furthermore, endothelial cells at the region between upper microposts gradually migrate toward the lower region, and form smooth and continuous microvessel wall at day 7.

under each condition. The microvessel boundary was strongly dependent on the HUVEC concentration.

Microvessels formed with 3×10^6 HUVECs/ml showed a rough and discontinuous boundary with the scattered small sprouts, and the microvessel was not perfusable. Microvessels formed with 9×10^6 HUVECs/ml had a continuous boundary with fewer, scattered, and small sprouts. However, the microvessels covered the majority of the area between the microposts at the upper region and exhibited a “rough” boundary. In contrast, microvessels formed with 6×10^6 HUVECs/ml was perfusable, and showed not only a continuous boundary without scattered small sprouts but also the smoothest boundary morphology with few sprouts protruding toward the upper region of the vessel channel.

Fig. 2(b) shows the success rate for microvessel formation for different HUVEC concentrations. Using 6×10^6 HUVECs/ml resulted in the highest success rate ($\sim 77\%$) in terms of obtaining smooth and continuous boundary morphology. Therefore, we concluded that the microvessels formed using 6×10^6 HUVECs/ml showed the most pertinent boundary morphology for our goal, and this condition was used in all further experiments.

Fig. 2(c) shows a series of micrographs during the microvessel formation using 6×10^6 HUVECs/ml condition. The HUVECs began to exhibit an elongated morphology with the small vacuoles 2 days after the seeding. At this time, the microvessels were not fully connected and no patent lumen was observed. HUVECs began to form a continuous lumen by day 4. The vessels began to merge to form a single continuous microvessel in the vessel channel. Furthermore, HUVECs in the upper region between the microposts began to migrate toward the lower direction, forming a flat and smooth microvessel boundary. The vessels fused to yield one fully lumenized microvessel by day 7, and HUVECs in the upper region between the microposts had migrated lower compared to those on day 4 to form the smooth microvessel boundary. The microvessel maintained its morphological characteristics for ≥ 14 days without a regression.

FIG. 3. Geometric and junctional characterization of the fully developed microvessel. (a) 3D projection and cross-sectional image of the microvessel shows patent lumen formation inside the microvessel in day 7. (b) DIC Micrograph of the microvessel wall and fluorescence micrograph of the same region with the introduction of 20 kDa FITC-dextran into the lumen. The fluorescence micrograph shows FITC-dextran containing microvessel without severe leakage at the vessel wall, showing the pertinent function of cell-cell junction. The experiment was performed with microvessels in day 7. (c) and (d) Junctional images of microvessel in day 7. Smooth and clear lines with elongated morphology of Claudin-5 and ZO-1 without wrinkled or disrupted region show pertinent junctional expressions similar to that *in vivo*.

Characterization of the microvessel

After the formation of the microvessel in day 7, CD31 (red) and nuclei (blue) were immunostained and imaged with a confocal microscope (Fig. 3(a)). Cross-sectional images of the microvessel at various positions revealed continuous patent three-dimensional lumens. The microvessel was composed of a clear lumen that connected both openings toward the media channels. To test the vessel integrity and continuity of the lumen, we introduced microbeads through the vessels. We confirmed that $\sim 94\%$ (101 of 108 chips) of the microvessels opened toward the media channels and could be perfused.

We introduced FITC-dextran solution (20 kDa) into the microvessel to verify the patency of the lumen, and visualized their behavior using a fluorescence microscopy. The FITC-dextran filled the microvessel lumen without severe focal leakage through the wall (Fig. 3(b)). The permeability coefficient of the microvessel was $1.58 \pm 0.32 \times 10^{-6} \text{ cm/s}$ (mean $\pm$ standard error, $n = 5$) as determined by a time-lapse acquisition of the FITC-dextran intensity profile. The permeability coefficient was in the range of those for other *in vitro* models of blood vessels,^{23–25} indicating that the microvessel expresses the appropriate cell-cell junctions.

We further analyzed the junctions by fluorescent immunostaining for claudin-5 and ZO-1 (Figs. 3(c) and 3(d)). The fluorescence images of two cell-cell junctions showed a smooth, clear elongated morphology without any disrupted or wrinkled positions, which are the basic characteristics of cell-cell junctions in normal blood vessels *in vivo*. We confirmed that the microvessels formed in this chip exhibited the cell-cell junctions similar to those *in vivo*, based on the permeability measurements and imaging of the junctions.

Cancer angiogenesis assay

We analyzed the angiogenic potential of cancer cells by using the microvessels with a smooth vessel wall. After formation of the microvessels in the channel (day 7–8), we introduced U87MG, a glioblastoma cell line with high angiogenic potential, and a fibrin mixture

into the upper channel, while the bridge channel was filled with fibrin gel only. We regarded the microvessel as that pre-existing at the cancer site, whereas the U87MG cells in the perivascular region secrete angiogenic factors to induce production of angiogenic sprouts toward the microvessels. We tested three different conditions at the upper channel: Only fibrin gel without cell as a control, and 5×10^6 U87MG/ml with or without bevacizumab (bev), which is an anti-VEGF antibody used widely for targeting angiogenesis. We analyzed the angiogenic sprouting from the microvessels and calculated the mean values of six chips per condition.

Three days after introduction of U87MG cells into the chip, we imaged the microvessels and quantified the number and coverage area of angiogenic sprouts under each condition. As shown in Fig. 4(a), most of the microvessels without U87MG cells showed few angiogenic sprouts. Furthermore, most microvessels treated with bevacizumab had a flat boundary without angiogenic sprouts. In some bevacizumab-treated samples, we observed some sprouts regressed after three days of cancer cells and bevacizumab introduction (Fig. 4(a)). We postulate that this was due to the anti-angiogenic effect of bevacizumab; however, a more in-depth analysis is required. Compared to these results, microvessels with U87MG cells showed a significant number of angiogenic sprouts (Fig. 4(b)). Enlarged three-dimensional confocal image of the vessel sprouts shows clear sprout formations from the vessel wall.

We next quantified the number and coverage area of sprouts. The microvessels with cancer cells exhibited markedly increased sprout numbers and coverage areas compared to the control (Figs. 4(c) and 4(d)). Furthermore, bevacizumab treatment attenuated the angiogenic potential of the U87MG cells, drastically decreasing the number and coverage area of the microvessel sprouts. These trends of the sprout induction by cancer cells and sprout attenuation by anti-

FIG. 4. Micrographs and quantification of the cancer angiogenesis assay. (a) Comparison of the microvessel wall before and three days in control experiment, and the cancer injection with bevacizumab treatment. No significant angiogenic sprouts are seen for both conditions. (b) Comparison of the microvessel wall before and three days after the cancer injection. Angiogenic sprouts from the microvessel wall are formed toward the upper channel, stimulated by the secretion of cancer cells. 3D confocal image (CD31) also clearly shows the angiogenic sprouts stimulated by the cancer secretion. (c) and (d) Quantification of coverage area and numbers of sprouts. Cancer cells induce significant amount of sprouts from the microvessel, and bevacizumab treatment drastically reduces the coverage area and the number of cancer sprouts, showing the anti-angiogenic potential of the bevacizumab for the cancer therapy ($***p < 0.0005$). Two independent experimenters performed the counting for the data in the graphs. Error bars represent SEM.

VEGF treatment are in agreement with previous *in vivo* reports,^{22,26} and demonstrate that this microvessel model is appropriate for the assessment of drugs targeting cancer angiogenesis.

Cancer intravasation assay

We next used the microvessel as a pre-existing blood vessel for assessment of transendothelial migration of MDA-MB-231, an aggressive metastatic breast cancer cell line.^{7,27,28} As shown in Fig. 1(d), cancer cells attached to the interface between the fibrin gel and bridge channel, and were incubated for 2–3 days to allow them to migrate toward and penetrate into the microvessels. Growth factor-free media (EBM-2) was introduced in the upper channel during the incubation. By supplying growth factor-free media (EBM-2) at the upper channel, we generated increasing gradient of growth factor concentration toward the vessel channel. Stimulated by the growth factor gradient, the cancer cells migrated to the microvessel site at the vessel channel. Fig. 5(a) shows fluorescence and differential interference contrast micrographs of a MDA-MB-231 cancer cell (green) in the process of intravasation through the microvessel wall (red). Fig. 5(b) shows a fluorescence and differential interference contrast micrograph of a MDA-MB-231 cancer cell (green) in the process of migration toward the microvessel wall. The image was acquired at one day after the cancer introduction into the bridge channel. After incubating for three days, we could see several intravasated cancer cells into the vessel lumen. We performed three-dimensional imaging of the microvessel with an intravasated cancer cell. The

FIG. 5. Micrographs and quantification of the cancer intravasation assay. (a) Micrographs of a cancer cell trans-migrating through the microvessel wall. (Red: CD31, Green: MDA-MB-231, and Blue: Nuclei) Fluorescence and DIC micrographs show that the cancer cell is embedded into the microvessel wall. (b) Micrograph of cancer cells migrating toward the microvessel wall (Green: MDA-MB-231 and Blue: Nuclei). The image was acquired two days after the cancer introduction into the bridge channel. (c) The cross-sectional images clearly show the cancer cell intravasated through the vessel wall and located in the vessel lumen (White: CD31 and Green: MDA-MB-231). (d) and (e) Micrograph of the VE-Cadherin expressed at the cell-to-cell junction in the microvessel. Compared to the microvessels in normal condition (d), VE-cadherin in TNF- α treated microvessel shows disrupted and wrinkled morphology (e), showing the junctional disruption by the effect of TNF- α . (f) Comparison of the intravasation rate between control and TNF- α treated microvessel. Compared to the control experiment, TNF- α treated microvessel showed a significantly higher rate of cancer intravasation, showing the effect of the TNF- α to the junctional function of the microvessel and cancer intravasation (* $p < 0.05$ compared to control). Error bars represent SEM.

cross-sectional images clearly show the intravasated cancer cell that is attaching its body on the inside of the vessel wall (Fig. 5(c)). We manually counted the number of cancer cells intravasated into the microvessels by analyzing three-dimensional images at day 3.

In this assay, the microvessels were treated with 5 ng/ml TNF- α 24 h before the introduction of the cancer cells and compared with the rate of cancer intravasation to that of the microvessels which was not treated with TNF- α (control). We first determined the effect of TNF- α on junctional expression by endothelial cells. The adherens junction VE-cadherin in the TNF- α treated microvessels exhibited disrupted continuity and wrinkled morphology compared to control microvessels (Figs. 5(d) and 5(e)). The permeability of TNF- α -treated microvessels over 24 h was $2.22 \pm 0.67 \times 10^{-6}$ cm/s, (mean $\pm$ standard error, $n=5$ chips), which was ~ 1.4 -fold higher than under the normal condition. These results confirm the effect of TNF- α on junctional disruption of endothelial cells, and are in agreement with previous reports.^{29–31}

We further analyzed the effect of TNF- α on cancer cell intravasation. We compared the rate of intravasation with or without TNF- α treatment, ($n=7$ chips per condition). The rate of cancer intravasation of TNF- α -treated microvessels was ~ 3 -fold higher than that of the control (Fig. 5(f)). This suggests that TNF- α treatment exerted marked effects on cancer cell intravasation, in agreement with previous reports.^{15,32,33}

DISCUSSION

We developed a microfluidic chip for assays of cancer angiogenesis and intravasation. Many previous angiogenesis or intravasation assays use an endothelial monolayer attached to a hydrogel or porous membrane.^{15,25,34} Our assay involves use of a microvessel formed by natural vasculogenic processes as a pre-existing vessel for cancer cells; thus the model mimics the *in vivo* conditions with higher similarity.

This chip design has two main advantages over previous chips developed by our group regarding vascular structural engineering.^{14,19} First, this chip facilitated introduction of diverse cell types into the perivascular region after formation of microvessels in the vessel channel. This was achieved by locating LF channels on the same side (lower region), while the upper region was exposed to an empty channel, which was filled with other cell types after formation of the microvessels. Second, this chip resulted in generation of a smooth and continuous vessel boundary, which is important for further research. If the vessel boundary is overly bumpy and complex, analysis of the cancer angiogenesis and intravasation data would be laborious and inaccurate. Furthermore, when sprout tips remained at the vessel wall, as under the 3×10^6 /ml condition (Fig. 2(a)), occasional focal leakage occurred at the tip, which indicated loose cell-to-cell junctions. This may also affect the results of experiments.

The mechanism underlying the generation of a smooth and continuous vessel boundary was not determined in this study. However, we identified two key factors for generating a smooth and continuous vessel boundary. First, the channel that contacts the vessel boundary should not be filled with gel or media. The bridge channel must contain only air during microvessel growth. This prevents the generation of growth factor gradients from the bridge channel, and prevents most of the endothelial cells from migrating or generating sprouts toward that empty channel. At the same time, media is supplied from the media channels, which led to migration of the endothelial cells toward the lower region, closer to the media channels. Therefore, the endothelial cells at the gap between microposts tend to migrate toward the lower direction and form a smooth vessel boundary (Fig. 2(c)).

Second, optimizing the HUVEC concentration was important for generating a smooth vessel boundary. A smooth boundary was not formed at low HUVEC concentrations. As under the 3×10^6 /ml condition (Fig. 2(a)), endothelial cells formed a thicker vessel in the lower region, whereas they were scarce in the upper region of the vessel channel. We speculated that this was due to migration of endothelial cells toward the media supply, and cells in the upper region of the vessel channel became scarce because the number of endothelial cells was insufficient to fill the vessel channel from the lower to the upper region. In contrast, if the HUVEC concentration was too high, excess endothelial cells were present in the vessel channel, which inhibited

migration of endothelial cells and generation of a smooth vessel boundary, as under the $9 \times 10^6/\text{ml}$ condition (Fig. 2(a)). Therefore, the optimal HUVEC concentration was $6 \times 10^6/\text{ml}$, which was used in all experiments.

The system described in this work shows promising junctional characteristics with pertinent three dimensionality. However, there are several potential limitations in terms of physiological similarity to the blood vessels *in vivo*. Although the geometry that locates stromal cells at the lower side allows straight and smooth vessel boundary, there could be non-physiologic gradient of growth factors secreted from LF, through the width of the vessel channel during the vessel maturation. The HUVECs that are close to the media channels will be sufficiently supplied by the growth factors, while the HUVECs located far from the media channels could not be. They will be equally supplied by the factors after the vessel opens their lumen to the media channels, but the non-physiological gradient formation during the vessel maturation can affect the physiological characteristics of the microvessels. Furthermore, the microvessels were solely grown without pericytes or smooth muscle cells, which contribute to the stabilization and normal function of capillaries and larger venules, respectively.

In the conventional transwell models, cancer cells are seeded at the surface of the endothelial monolayer for testing transendothelial migration of them. Compared to that cancer cells in this platform should migrate toward the microvessel wall through the fibrin gel before performing transendothelial migration. Therefore, this model has much lower rate of transendothelial migration compared to that in conventional transwell models.^{9,35} However, it is not a low value compared to *in vivo* or *in vitro* published results that require cancer migration through the ECM before the transendothelial migration.¹⁵

To demonstrate the utility of the metastasis chip, we established two assays using microvessels generated by natural vasculogenic process. Cancer angiogenesis and its inhibition by the treatment of anti-VEGF (bevacizumab) were successfully reproduced. Cancer intravasation, together with its modulation by the treatment of TNF- α , was also recreated using this model. Although further investigation is required, we speculate that this model will facilitate investigation of cancer angiogenesis and intravasation due to its use of a more realistic method of generating the vascular structure, whereas other models use an artificial coating of endothelial cells on a hydrogel or porous membrane. Comparing this system with other pre-established *in vitro* models with deeper biology will be a topic for future investigations. This model has potential to be applied for various studies, including cancer cell or leukocyte extravasation, mechanotransduction of endothelial cells to the intraluminal flow. By integrating labeled-cell separation techniques, selective collection of the intravasated cancer cells and further analyze of them will also be possible. The robustness and reproducibility of this microfluidic platform for investigation of cancer-EC interactions will facilitate further research in fundamental cancer biology and drug screening.

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