Genes to Cells

In situ electrical monitoring of cancer cells invading vascular endothelial cells with semiconductor-based biosensor

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Cellular dynamics is very closely related to ionic behaviors, most of which have been hardly monitored in real time, whereas semiconductor-based biosensors have the unique advantage of direct detection of ionic charges in a real-time and noninvasive manner. In this study, we monitored the invasion process of cancer cells into the vascular endothelial layer in real time by a label-free method using a field-effect transistor (FET) biosensor. Endothelial cells were cultured on the sensing surface of the FET gate, to form a basement membrane between the endothelial cells and the sensing surface. When invasive cancer cells (HeLa cells) approached the endothelial cell-coated gate FET biosensor, a change in the surface potential was clearly detected using the FET biosensor. This is because HeLa cells, which invaded the endothelial cell layer, reduced the molecular charge density in the basement membrane by decomposing it. A platform based on the cell-coated gate FET biosensor is suitable for real-time and noninvasive monitoring of cellular dynamics based on intrinsic ionic charges.

Introduction

Metastatic cancer cells, which flow in blood, adhere to vascular endothelial cells inside blood vessels, resulting in the invasion of cancer cells and metastasis. Measurement methods for analyzing invasion processes of cancer cells have contributed to not only the elucidation of the mechanism of metastasis but also the realization of early diagnosis of cancer. In fact, various biochemical processes in metastatic cancer cells have been clarified, such as their interaction with vascular endothelial cells, the role of adhesive molecules and cancer cell extravasation from blood vessels (Chambers *et al.* 2002; Eger & Mikulits 2005; Mehlen & Puisieux 2006; van Zijl *et al.* 2011). However, no measurement method has been developed to analyze the metastasis processes in a real-time and label-free manner.

To solve this problem, biosensors are being developed for *in situ* monitoring of cellular activities. As an example of such biosensors, semiconductor-based biosensors enable the detection of cellular functions in a real-time, label-free and noninvasive manner (Fromherz *et al.* 1991; Peitz *et al.* 2007; Sakata & Miyahara 2008; Sakata *et al.* 2012, 2013), based on the detection of changes in ionic charge density induced by cellular activities. In our previous works, field-effect transistor (FET) biosensors monitored some cellular functions in real time, such as cellular respiration and programmed cell death (apoptosis), on the basis of ionic charges (Sakata & Miyahara 2008; Sakata *et al.* 2012, 2013). Such ionic charges have to be selectively detected around a cell/sensor interface depending on the cellular functions of interest. To analyze specific ionic behaviors on the basis of cellular functions, therefore, an appropriate signal transduction material and structure should be developed around the cell/sensor interface. In particular, we propose a biologically induced interface composed of living cells as the interfacial material and simulate the interaction between cancer cells and endothelial cells in blood on the sensor surface. When vascular endothelial cells are cultured on a sensor surface to form a biological and signal transduction interface, the interaction of cancer cells with the endothelial

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cells will be converted into electrical signals to the sensor.

In this study, we developed a simple structure of blood vessels on a FET biosensor and monitored the invasion process of cancer cells (HeLa cells) into these vessels with vascular endothelial cells [human coronary artery endothelial (HCAE) cells] in a real-time and noninvasive manner using the FET biosensor. For this purpose, the role of a basement membrane, which separates endothelial cells from the underlying connective sensor, in generating electrical signals was investigated.

Results and discussion

Concept of biologically induced interface for FET biosensor

Figure 1 shows the detection concept of the VEC-FET biosensor. The detection principle of the FET biosensor is based on the potentiometric detection of changes in charge density at the gate insulator, on which specific binding between the target and probe molecules occurs for molecular recognition (Sakurai & Husimi 1992; Sakata & Miyahara 2006; Sakata *et al.* 2009; Rothberg *et al.* 2011). In the case of the VEC-FET biosensor, HCAE cells are cultured on the gate surface to form the signal transduction interface, which translate ionic changes to the FET by their interaction with or invasion of cancer cells, as shown in Fig. 1A. Actually, HCAE cells were cultured on the sensing surface of the gate and observed as live cells stained with calcein-AM fluorescent dyes, as shown in Fig. 1B. In particular, the basement membrane is generated on the gate surface by endothelial cells, which is a thin, fibrous, extracellular matrix (ECM) of tissue and composed of collagen IV, laminin, heparan sulfate proteoglycan and so on. In this study, Matrigel was also coated on the gate surface to form the basement membrane, which induced the adhesion of HCAE cells to the gate. The gate surface covered by HCAE cells was immersed in a culture medium together with a Ag/AgCl reference electrode, in which HeLa cells were given to monitor the invasion process. Basically, ionic or molecular charges at the gate interact electrostatically with electrons in silicon crystals across the thin gate insulator and induce electrical signals by the field effect, resulting in a change in I_D at the channel. Eventually, the changes in charge density at the gate are detected by the VEC-FET biosensor as a change in V_T at a constant I_D.

Role of basement membrane in signal transduction

The basement membrane is decomposed by collagenase secreted by cancer cells during the invasion process (Liotta *et al.* 1987). Therefore, the change in the electrical signals of the FET biosensors upon the

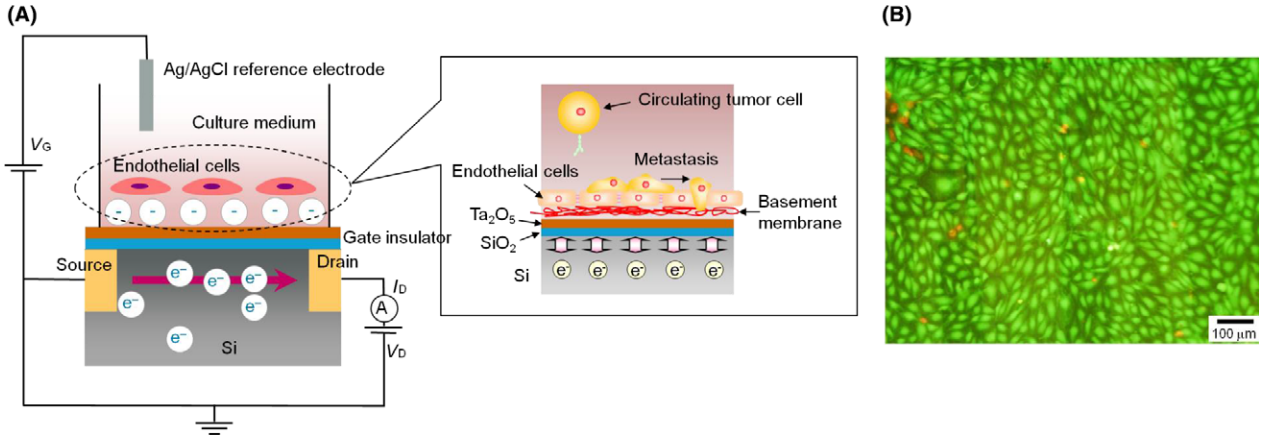

Figure 1 Schematic illustration of vascular endothelial cell-based field-effect transistor (VEC-FET). (A) Endothelial cells were cultured on the gate of the FET biosensor, to which cancer cells were added to investigate the invasion process. The basement membrane was composed of conventional Matrigel coated on the gate before cell culture and is secreted by endothelial cells. (B) Fluorescence image of human coronary artery endothelial (HCAE) cells stained by calcein-AM fluorescent dyes (green). The image shows that the HCAE cells were normal and alive on the gate of the FET biosensor, although a very small number of HCAE cells were dead and stained by propidium iodide (red). HCAE cells were cultured on the gate for 2 days.

decomposition of Matrigel coated on the gate by collagenase should be measured in cell-free experiments. Figure 2 shows ΔV_T in V_G-I_D electrical characteristics detected by the FET with Matrigel coated on the gate. Matrigel is often used as a basement membrane matrix in cell-free experiments and a mixture of ECM proteins (Orkin *et al.* 1977; Kleinman *et al.* 1982; Kleinman & Martin 2005). In particular, Matrigel is negatively charged in a solution because it contains heparan sulfate proteoglycan among others. As shown in Fig. 2A, V_T shifted by approximately 80 mV in the positive direction on the Matrigel-coated gate of the FET device, whereas V_T decreased and almost returned to the initial state after the administration of collagenase. However, ΔV_T was not fully recovered after collagenase treatment. This may be because Matrigel was not completely decomposed and a part of Matrigel remained on the gate. These changes were clearly observed, as shown in Fig. 2B. This is because the negatively charged Matrigel was coated on the gate surface and was decomposed by the enzymatic reaction (Fig. S1C in Supporting information). In this case, PBS was used as the measurement solution, whose ionic strength is much lower than that of the culture medium, which contained various salts and amino acids, glucose and various proteins as nutrients. This means that the molecular charges in Matrigel were more difficult to be

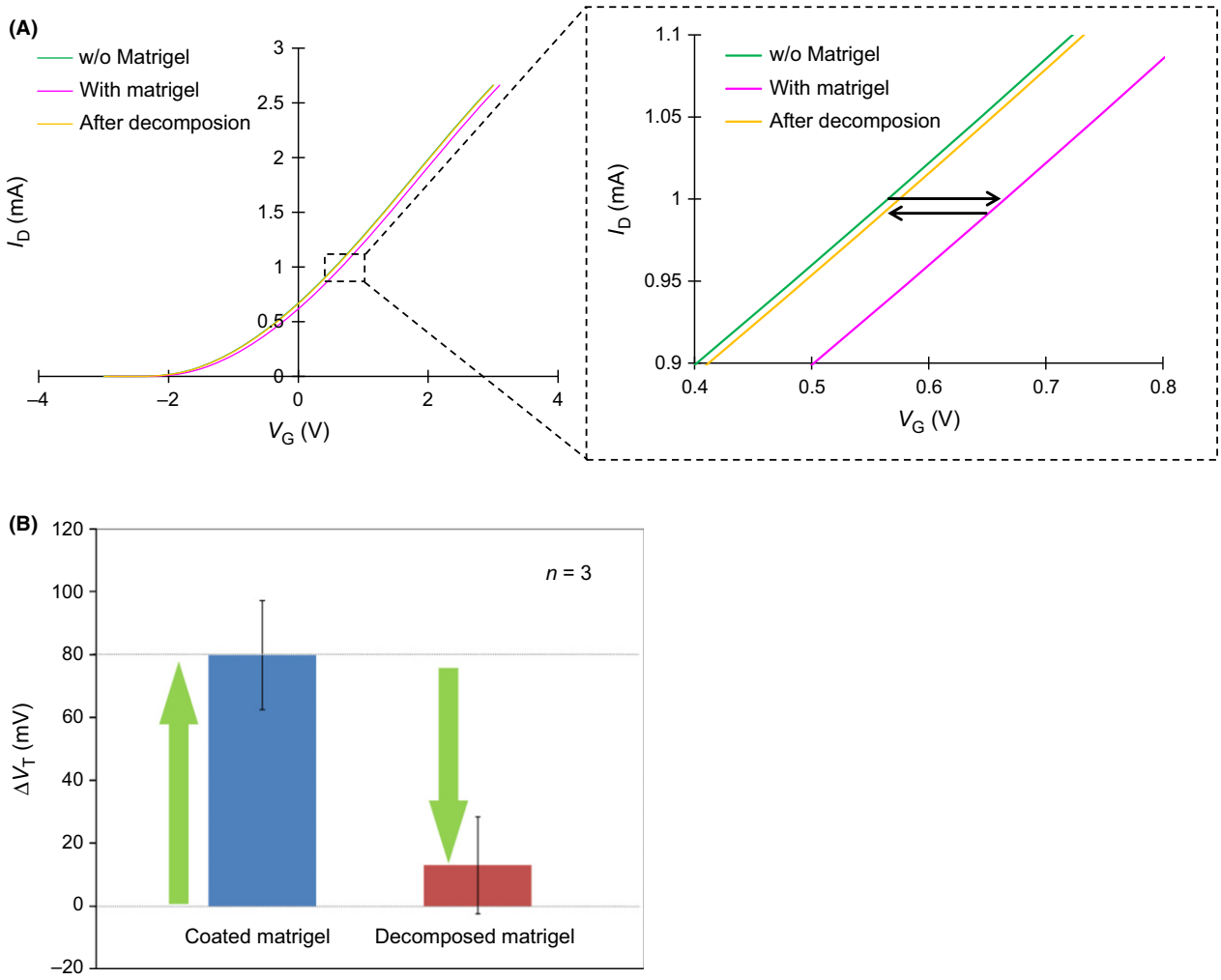

Figure 2 Electrical characteristics of FET biosensor with Matrigel. (A) Threshold voltage shift (ΔV_T) in gate voltage (V_G)–drain current (I_D) characteristic of FET biosensor detected by coating and decomposing Matrigel on the gate. (B) Change in V_G at constant drain current (I_D = 2 mA), that is, ΔV_T. The measurements were taken using three different sensors.

screened by counter ions in PBS than those in the culture medium, depending on the electric double-layer (EDL) structure (Debye & Hückel 1923; Israelachvili 1991; Maekawa *et al.* 2013); therefore, relatively larger shifts in the signals were obtained in PBS than in the culture medium (see the next section '*Real-time monitoring of surface potential for attachment of tumor cells with endothelial cells using VEC-FET*'). The measurement deviation of ΔV_T shown in Fig. 2 was contributed to the thickness of Matrigel. This is because Matrigels with different thickness would cause the change in the number of charges inside them. However, the deviation was not so large and the repeatability would be mostly maintained, because the coating of Matrigel was carried out under the same condition in this study.

Figure 3 shows the fluorescence images of actin filaments in HCAE cells and collagen IV in the basement membrane before and after adding HeLa cells. As shown in Fig. 3A, several irregular gaps between HCAE cells, which were structured by actin filaments, were observed after the addition of HeLa cells. Moreover, collagen IV was observed under endothelial cells before adding Hela cells, whereas it was partially decomposed 6 h after adding HeLa cells to HCAE cells, as shown in Fig. 3B. Thus, HeLa cells are considered to have decomposed the basement membrane by secreting the collagen IV-decomposing enzyme, which passed between the HCAE cells.

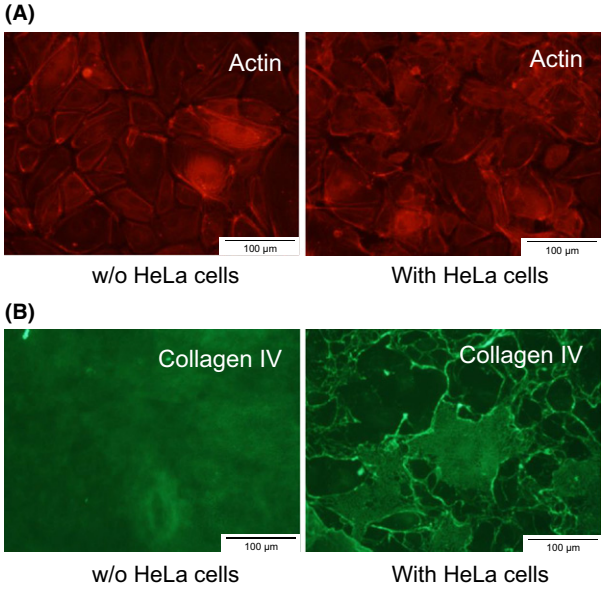

Figure 3 Fluorescence images of endothelial cells and basement membrane before and after adding HeLa cells. (A) Actin filaments in HCAE cells. (B) Collagen IV in basement membrane.

Real-time monitoring of surface potential for attachment of tumor cells with endothelial cells using VEC-FET

Figure 4 shows the change in surface potential after the interaction of HeLa (tumor) cells with the VEC-FET. Using the electrical circuit system shown in Fig. 4A, the surface potential of the VEC-FET can be monitored in real time. As shown in Fig. 4B at an initial stage of measurement time (0–0.2 h), both potentials of the VEC-FETs were normalized as almost zero, which made it easy to analyze the effect of added samples on the electrical signals. After the addition of sample solutions at approximately 0.2 h, the surface potentials showed temporarily negative changes for both sensors with and without tumor cells. The temporal negative shifts were due to the change in pH in their culture mediums. That is, the vascular endothelial cells had been already cultured on the gate (VEC-FET) before the addition of tumor cells, resulting in the change in pH (to an acid side) owing to the cellular respiration activity. When fresh culture mediums with or without tumor cells were added onto each VEC-FET, pH in both culture mediums would roughly return to the original pH; therefore, the surface potential showed temporarily the negative changes after the addition of culture mediums with or without tumor cells, because the initial potentials were also set as zero. Subsequently, ΔV_{out} gradually increased after introducing tumor cells onto the VEC-FET, which was caused by the decrease in the density of negative charges or the increase in the density of positive charges on the gate, whereas the surface potential for the VEC-FET without tumor cells kept the negative change, although it was very small. Considering signal shifts in Fig. 2 and the morphological properties shown in Fig. 3, the increase in ΔV_{out} is considered to have been induced by the decomposition of the basement membrane due to the invasion of tumor cells, corresponding to the decrease in negative charges. From the signal shifts, the basement membrane seemed to be gradually decomposed over 2–4 h. From the viewpoint of reaction time, the decomposition process of Matrigel induced by the addition of collagenase (Fig. 2) would be different from that of basement membrane caused by the invasion of HeLa cells (Fig. 4). This is because Hela cells took a few hours to adhere to and invade HCAE cells, whereas the addition of collagenase onto the Matrigel-coated gate

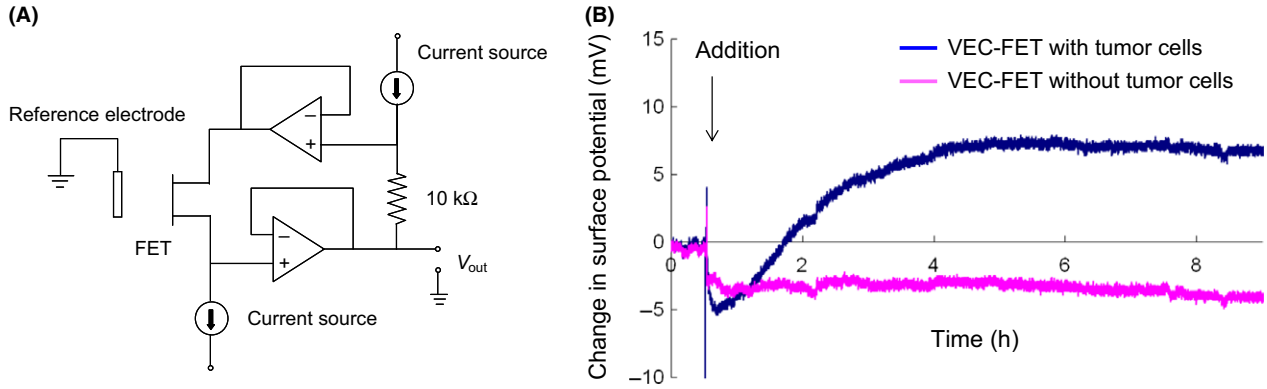

Figure 4 (A) Electrical circuit for measuring surface potential of VEC-FET sensor (Sakata *et al.* 2013). (B) Change in surface potential of VEC-FET measured for analysis of invasion of cancer cells. HeLa cells were added onto the VEC-FET biosensor for *in situ* monitoring of invasive cancer cells. However, the culture medium without HeLa cells was added onto the VEC-FET as the control sensor.

surface showed the quick response by the decomposition of Matrigel using the FET biosensor, as shown in Fig. S2 (in Supporting information). During this time, the total change in V_{out} was approximately 10 mV, which was smaller than ΔV_T determined from the decomposition of Matrigel, as shown in Fig 2. This can be explained by detection limit based on the Debye length, as follows. Biomolecular charges at the gate electrode/electrolyte solution interface were detected using the FET biosensor, depending on the Debye length at the interface (Sakata & Miyahara 2007; Wu *et al.* 2015). The Debye length is estimated from the charge distribution at a solid/liquid interface, which corresponds to the distance from a substrate on the basis of Stern and diffusion layers indicating EDL and varies according to the ionic strength in a measurement solution (Debye & Hückel 1923; Israelachvili 1991; Maekawa *et al.* 2013). In the case of the FET biosensors, the amount of biomolecular charges within the Debye length at the gate is very closely related to the quantitative analysis of biomolecular recognition events. In this study, the Debye length was at most less than a few nanometers because various ionic molecules were included in the culture medium, which mostly shielded the molecular charges of ECM at the gate surface. This means that the electrical signal of the FET biosensor is caused only by the change in ionic or molecular charges extremely close to the gate surface. What existed extremely close to the gate surface in this device was basement membrane, the thickness of which was in the range of nanoscale to submicron. Therefore, the signal shift in shown in Fig. 4 is considered to have been caused by the dissociated components with charges extremely close to the gate surface, although it might slightly include the change in molecular charges at the cell membrane due to the attachment of HeLa cells to the gate surface or the detachment of HCAE cells from the gate surface. On the basis of such ionic behaviors around the cell/gate interface, the invasion process of tumor cells into blood vessels with vascular endothelial cells was monitored in a real-time and noninvasive manner.

Conclusions

In this study, we showed that a FET biosensor was capable of monitoring the invasion process of cancer cells into endothelial cells in a real-time and noninvasive manner. This is because the molecular charges of the basement membrane were detected with high sensitivity on the basis of the principle of the field effect, which was confirmed by the electrical measurement and fluorescence imaging of the decomposition of ECM contained in the basement membrane. A platform based on the FET biosensor is suitable for *in situ* monitoring of cellular dynamics in a real-time and noninvasive manner, which is expected to contribute to the elucidation of cellular behaviors such as cell–cell interactions *in vivo*.

Experimental procedures

Coating and decomposition of Matrigel

A glass ring with a diameter of 10 mm (500 μL) was fixed on a substrate excluding a gate sensing area using polydimethylsiloxane (PDMS) (Fig. S1A in Supporting information). Matrigel (Corning), which is a reconstituted basement membrane

extracted from Engelbreth Holm Swarm (EHS) mouse sarcoma and contains laminin, collagen IV, entactin, heparan sulfate proteoglycan (perlecan), TGF-β, epidermal growth factor, insulin-like growth factor, fibroblast growth factor, tissue plasminogen activator and other growth factors (Orkin *et al.* 1977; Kleinman *et al.* 1982; Kleinman & Martin 2005), was diluted ten times with phosphate-buffered solution (PBS; 25 mM Na_2HPO_4 and 25 mM KH_2PO_4; Wako). The diluted Matrigel was dipped to be 50 μL/cm^2 at 4 °C for 30 min in the glass ring well, where the gate electrode of the FET biosensor is set. Then, the supernatant of the solution was removed and the remaining sample solution on the gate was dried at room temperature in a clean bench. The Matrigel-coated FET biosensor was used for electrical measurements and cell cultures (Fig. S1B in Supporting information). For the analysis of the decomposition of Matrigel, 0.2% collagenase (Wako) was added to the Matrigel-coated FET biosensor (Fig. S1C in Supporting information). PBS was used as the measurement solution after the surface treatment.

Electrical measurement using FET device

We used a silicon-based n-channel depletion-mode FET with a Ta_2O_5/SiO_2 (100/50 nm) layer as a gate insulator with a width (W) and length (L) of 340 and 10 μm, respectively. The Ta_2O_5 thin film was used as a passivation layer to prevent leakage currents in a buffer solution. The gate voltage (V_G)–drain current (I_D) electrical characteristics were measured using a semiconductor parameter analyzer (B1500A; Agilent). A change in V_G in the V_G-I_D electrical characteristics was estimated as a threshold voltage (V_T) shift, which was evaluated at a constant I_D of 1 mA and at a constant of drain voltage (V_D) of 2 V. PBS was prepared as a measurement solution for the analysis of Matrigel on the gate, and a culture medium (see *2.3.*) was used for the measurement of cellular behaviors. A Ag/AgCl reference electrode with a KCl solution was connected to the measurement solution through a salt bridge. The size of gate was enough to detect signals from cells with a diameter of approximately 10 μm. In particular, W/L at the channel was designed to be adequate for the sensitivity of I_D for V_G. Additionally, the thickness of gate insulating layers was enough to prevent leakage currents through the insulating layer caused by bias applying and invasion of ions in solutions.

Cell culture on VEC-FET biosensor

HCAE cells (PromoCell) were precultured on a cell culture dish at 37 °C in 5% CO_2 in an incubator system. The endothelial cell growth medium MV2 (PromoCell), which contains 0.05 mL/mL fetal calf serum, 10 ng/mL epidermal growth factor (human recombinant), 10 ng/mL basic fibroblast growth factor, 20 ng/mL insulin-like growth factor, 0.5 ng/mL vascular endothelial growth factor (human recombinant), 1 mg/mL ascorbic acid and 1 mg/mL hydrocortisone, was prepared as the culture medium. HCAE cells of approximately 1×10^5 cells/mL were seeded in the glass ring well (500 μL) so that they were confluently cultured on the Matrigel-coated gate electrode. The FET biosensor with HCAE cells, that is, the vascular endothelial cell-based FET (VEC-FET), was set up for electrical measurements at 37 °C in the incubator system, which included a microscope. After confirming that signal traces had almost become steady, 10 μL of the culture medium including HeLa cells (5×10^6 cells/mL) was added to the gate surface of the VEC-FET biosensor, then the time of addition was defined as the starting point (0 h) for electrical measurements. To examine the effects of background noises, such as changes in temperature and ion concentration, on electrical signals, 10 μL of the culture medium without HeLa cells was added to the gate surface of another VEC-FET biosensor. We set a standard signal drift of less than a few mV per hour as the baseline of the steady potential.

Microscopic observation

A red fluorescent conjugate (Acti-stain 555 phalloidin; Cytoskeleton) was used to observe actin filaments in HCAE cells on a substrate. Collagen IV was stained to observe the basement membrane by immunofluorescence microscopy. Diluted polyclonal rabbit anti-human collagen IV serum diluted 1 : 100 was used as the primary antibody (Euro-Diagnostica AB), and the cells were treated with 10 μg/mL Alexa Fluor 488-labeled goat anti-rabbit IgG (Invitrogen) for 60 min. Cells on cover slips were prepared for confocal microscopic observation (Carl Zeiss LSM510 META NLO). The microscopic observations were carried out 0 h and 6 h before and after HeLa cells were administered to HCAE cells, which were precultured on the gate for 2 days before their interaction with HeLa cells.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Conceptual structure of FET biosensor with Matrigel for electrical measurements.

Figure S2 Real-time monitoring for Matrigel degradation by collagenase using FET device.