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Research Article

Mucin (MUC5AC) expression by lung epithelial cells cultured in a microfluidic gradient device

We have developed a microfluidic gradient device for controlling mucin gene expression of NCI-H292 epithelial cells derived from lung tissues. We hypothesized that gradient profiles would control mucin gene expression of lung epithelial cells. However, it was not possible to generate various stable gradient profiles using conventional culture methods. To address this limitation, we used a microfluidic gradient device to create various gradient profiles (*i.e.* non-linear, linear, and flat) in a temporal and spatial manner. NCI-H292 lung epithelial cells were exposed to concentration gradients of epidermal growth factor in a microfluidic gradient device with continuous medium perfusion. We demonstrated an effect of gradient profiles on mucin expression of lung epithelial cells cultured in the microfluidic gradient device. It was revealed that NCI-H292 lung epithelial cells exposed to the flat gradient profile of the epidermal growth factor exhibited high expression of mucin as compared with cells exposed to non-linear and linear gradient profiles. Therefore, this microfluidic gradient device could be a potentially useful tool for regulating the mucin expression of lung epithelial cells exposed to chemokine gradient profiles.

Keywords:
Concentration gradient / Lung epithelial cell / Microfluidics / Mucin expression
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1 Introduction

Mucin hypersecretion in conjunction with goblet hyperplasia/metaplasia is an important pathophysiologic feature of chronic airway inflammatory diseases, including asthma, chronic obstructive pulmonary disease, and cystic fibrosis [1]. Although appropriate mucin production creates homeostasis for the conducting airways by forming a physical boundary to prevent entry of microorganisms and particles into the airways, mucin overproduction leads to exacerbation of these diseases and represents a major cause of death in these diseases. Among 20 known mucin genes, MUC5AC is a major mucin in the human respiratory tract and serves as a specific marker of goblet cells [2–4]. MUC5AC is produced in response to a wide variety of stimuli, including epidermal growth factor (EGF) receptor ligands, inflammatory cytokines (*i.e.* tumor necrosis factor, interleukin (IL)-1β, IL-4, and IL-13), neural elastase, air-pollutants, reactive oxygen species, and bacterial products [5–12]. Control of MUC5AC gene expression may provide a feasible treatment option for airway inflammatory diseases.

The concentration gradient is of great interest with respect to controlling biological and clinical processes, such as cancer metastasis and the immune response [13–16]. Although conventional gradient generators (*i.e.* Boyden chamber) have been used to investigate the effect of concentration gradients on cellular behavior [17–19], they are not able to create stable concentration gradients or various gradient profiles in a single platform. To address these limitations, microfluidic gradient devices have been previously developed to control cellular behavior [20–30]. Using these devices, the cells are exposed to stable concentration gradients and gradient-responsive cell fate can be monitored in a real-time manner. Recently, a microfluidic-vacuum device containing a fluidic channel and vacuum network channel has been generated [31]. This microfluidic device has some advantages; it does not require an inlet port for cell seeding, because the vacuum allows reversible sealing between the microfluidic device and the substrate.

Microfluidic culture platforms have been previously used to culture lung cells and regulate lung functions [32–35]. For example, fluidic mechanical stress has been

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Abbreviations: EGF, epidermal growth factor; **FBS,** fetal bovine serum; **IL,** interleukin

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generated in a microfluidic airway device to study lung injury at the cellular level [32]. This microfluidic airway system enabled the generation of an air–liquid two-phase interface that consisted of an upper air channel, lower medium flow channel, and a sandwiching porous membrane where lung epithelial cells were cultured. It demonstrated the effect of liquid plug movement on mechanical lung injury, showing that the most injured cells were observed in the microchannel where liquid plugs were ruptured. Another study used a serpentine-based microfluidic gradient device for analyzing resistance of lung cancer cells to chemotherapy [33]. A concentration gradient of A23187, an inducer of glucose-regulated protein-78, was generated in a microfluidic device and expression of glucose-regulated protein-78 was shown to be increased by an increased concentration of A23187. In contrast, the apoptosis of lung cancer cells treated with an anticancer drug (*i.e.* VP-16) was decreased with increasing A23187 concentration. Furthermore, a microfluidic culture device that can generate mechanical strain has been recently developed to regulate lung functions [34]. To create a barrier between alveolar epithelium and capillary endothelium, a sandwiching polymeric membrane where epithelial cells and endothelial cells were cultured was used in a microfluidic device. This provided a model of pulmonary nanotoxicology, showing that the breathing motion (10% mechanical strain at 0.2 Hz) enhanced the cellular uptake of nanoparticles across the alveolar–capillary barrier. Time-lapse images also showed that neutrophils transmigrated through the alveolar–capillary barrier. However, previous microfluidic approaches did not consider the effect of various gradient profiles on the gene expression of lung epithelial cells. We first cultured NCI-H292 lung epithelial cells in a microfluidic gradient device and analyzed the effect of gradient profiles on the mucin expression. We hypothesized that gradient profiles of chemokines would enable the control of mucin gene expression after cell starvation. To investigate gradient-based mucin expression, various gradient profiles (*i.e.* non-linear, linear, and flat) were generated inside a single microfluidic device in a temporal and spatial manner. Therefore, this microfluidic gradient device could be a powerful tool for the culture of lung epithelial cells and analysis of the effect of gradient profiles on mucin expression.

2 Materials and methods

2.1 Microfabrication of a microfluidic gradient device

We developed a microfluidic gradient device that was consisted of a vacuum network channel and a winding-shaped fluidic channel with three inlets, as described previously (Fig. 1A) [31]. Briefly, SU-8 50 (Microchem, USA) photoresist was spin-coated onto a silicon wafer to make 100 μm thick microchannels. After the photolithography process, PDMS was molded in a photoresist-patterned silicon master. Inlets and outlets of the PDMS microfluidic device were punched using a sharp puncher and the device was sterilized by oxygen plasma (Femto Scientific, Korea) and ultraviolet light.

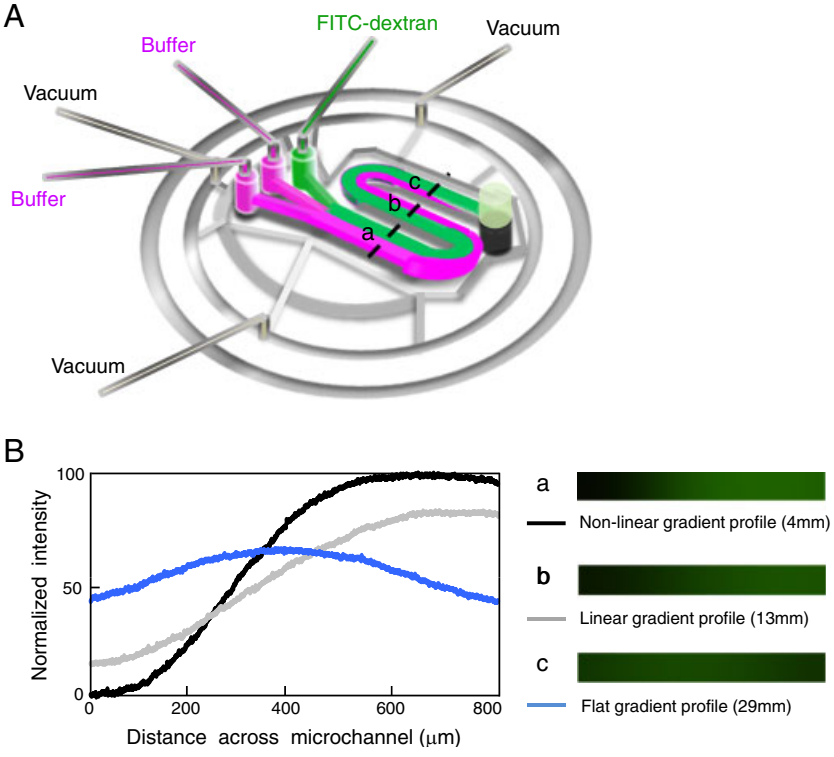

Figure 1. A microfluidic gradient device used to generate various concentration gradient profiles. (A) Schematic diagram of a microfluidic gradient device containing a winding-shaped fluidic channel and a vacuum network channel. Buffer was perfused into two left inlets and fluorescent dye (FITC-dextran) was infused into the right inlet. A vacuum was applied into vacuum network microchannels through three vacuum inlets for reversible attachment between the PDMS microfluidic device and the 6-well plate. (B) Characterization of gradient profiles in a microfluidic gradient device. Gradient profiles of FITC-dextran were measured at a distance of 4, 13, and 29 mm from the merging point of the three inlets, showing non-linear (a), linear (b), and flat (c) gradient.

2.2 Experimental setup

When vacuum was applied into the vacuum network channel, the PDMS microfluidic device and the 6-well plate were reversibly sealed. The liquid encapsulated within the vacuum network channel was completely removed, because vacuum was continuously applied through vacuum network channel. After reversible sealing between vacuum network channel of the microfluidic device and 6-well plate, culture medium was subsequently infused into a winding-shaped fluidic channel using a syringe pump. Furthermore, to analyze the mucin expression of lung epithelial cells, vacuum was removed from the vacuum network channel and the microfluidic device was then peeled off from the 6-well plate for further immunocytochemistry.

2.3 Characterization of gradient profiles and image analysis

To characterize the concentration gradient profiles in the microfluidic device, FITC-dextran (10 KDa, Sigma, USA) was infused into a right inlet and buffer solution was infused into a left and a middle inlet of the microfluidic device. The solutions were infused into a microchannel using a syringe pump (0.07 μL/min pumping speed; Harvard Instruments, USA). Fluorescent images of FITC-dextran were taken using an inverted microscope (Olympus, IX71, Japan), and gradient profiles (*i.e.* non-linear, linear, and flat) of FITC-dextran were analyzed using Image J software. We used a molecular weight (10 kDa) of FITC-dextran that was similar to the molecular weight of EGF (6 kDa). The normalized fluorescent intensity profiles of concentration gradients were obtained from fluorescent images of FITC-dextran (Fig. 1B).

2.4 NCI-H292 cell culture and cell starvation

NCI-H292 cells, human lung mucoepidermoid carcinoma cells [36], were cultured in RPMI1640 medium containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. Cells (3×10^5 cells/mL) were seeded in 6-well plates and cultured overnight, then washed with PBS (Welgene,

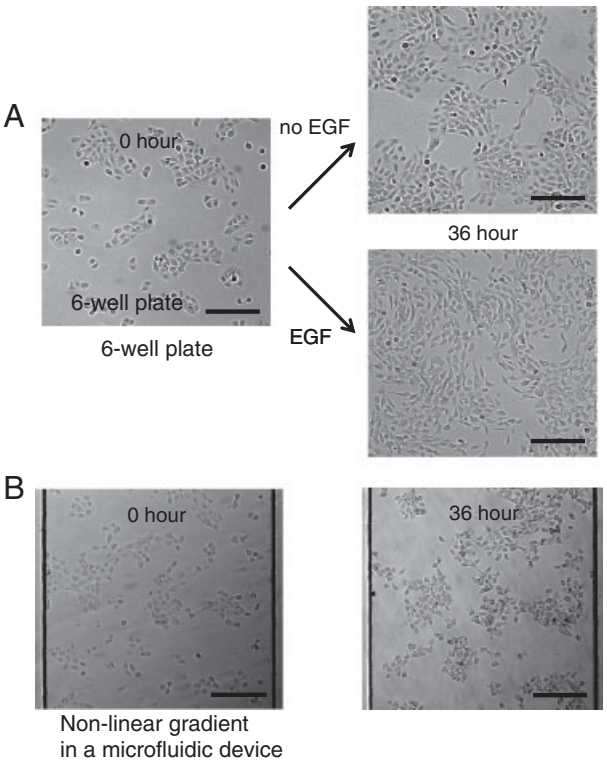

Figure 2. Phase contrast images of NCI-H292 cells cultured in a 6-well plate and the microfluidic device. The microphotographs of NCI-H292 cells that were cultured with and without EGF in a 6-well plate (A) and in the region of the non-linear EGF gradient profile of the microfluidic device (B). Scale bars are 200 μm.

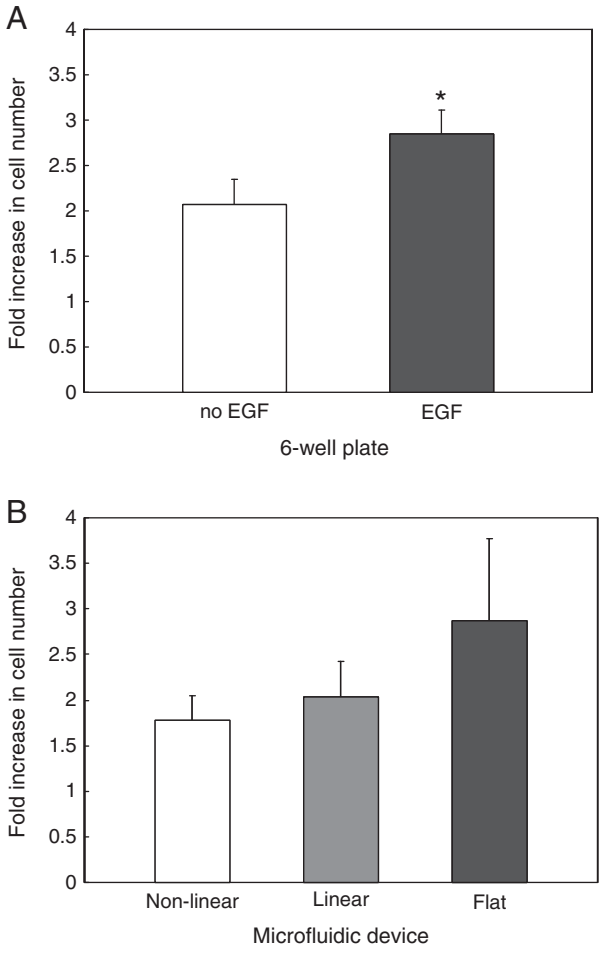

Figure 3. Proliferation of NCI-H292 cells cultured in a 6-well plate (A) and microfluidic gradient device (B). We calculated the fold increase in cell number, showing that the number of cells cultured for 36 h was divided by the initial cell number ($^*p < 0.05$). All quantification was performed three times at each gradient profile.

Korea) and were starved with serum-free medium for 5 h. The starved cells were washed with PBS and were subsequently cultured with RPMI1640 medium containing 2% FBS for 2 h. RPMI1640 medium containing 2% FBS and 10 ng/mL EGF (BD Pharmingen, USA) was infused into a right inlet of the microchannel, whereas RPMI1640 medium without serum and EGF was perfused into a left and a middle inlet of the microchannel.

2.5 Immunocytochemistry

To analyze MUC5AC protein expression in NCI-H292 cells exposed to EGF concentration gradients, the microfluidic gradient device was peeled off from the 6-well plate after removing the vacuum. The cells were fixed in 4% paraformaldehyde and permeabilized in TBS containing 0.1% saponin for 15 min at room temperature. The slides were treated with 0.3% H_2O_2–methanol for 20 min to block endogenous peroxidase, washed twice in TBS, treated with blocking solution for 20 min at room temperature, and incubated at 4°C overnight with anti-MUC5AC antibody (Chemicon, USA). After washing with TBS, the slides were treated with biotinylated goat anti-mouse IgG for 15 min at room temperature, and then with avidin–biotin peroxidase complex (Vector Laboratories, USA) for 15 min at room temperature. The color reaction was developed by staining with 3,3′-diaminobenzidine tetrachloride (Zymed Laboratories, USA). The reaction was terminated by washing in running water until a uniform brown color became visible on the section. Negative control slides were incubated with isotype-matched antibody. The immunostained samples were mounted in mounting solution and color images were taken using a camera (JENOPTIK, Germany).

3 Results and discussion

3.1 Microfluidic gradient device

We generated a microfluidic gradient device to culture lung epithelial cells (Fig. 1A). This microfluidic gradient device included a winding-shaped fluidic channel and a vacuum network channel. The dimensions of the microchannel of the device were 800 μm in width and 100 μm in height. Vacuum sealing through a vacuum network channel allowed a reversible bonding between the microfluidic gradient device and the 6-well plate in which cells had been grown. This microfluidic gradient device had several advantages over the previously reported microfluidic vacuum device [31]. First, it allowed infusion of medium into the microchannel without any leakage, because a strong vacuum, which was created by multiple vacuum inlets, was applied into the vacuum network channels. In contrast, the previously reported device [31] has only a single vacuum inlet, which was not able to provide sufficient vacuum pressure to maintain stable infusion-based gradients. Second, owing to a long winding-shaped microchannel (35 mm channel length), we could generate multiple gradient profiles inside a single microfluidic device in a temporal and spatial manner.

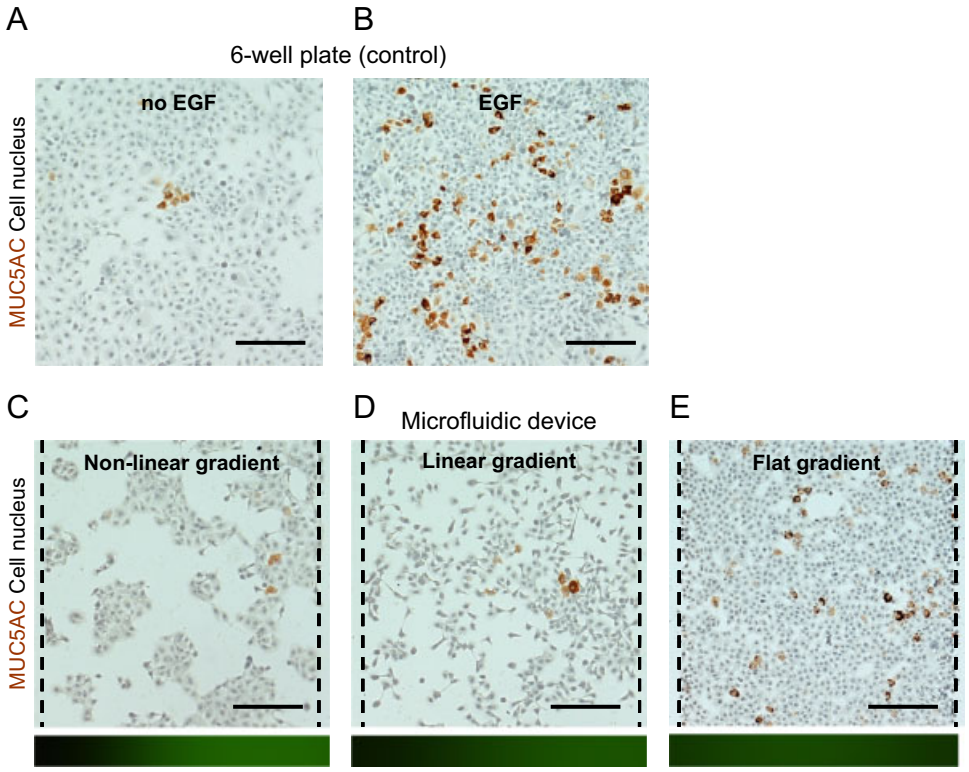

Figure 4. MUC5AC-positive NCI-H292 cells cultured in a 6-well plate (A and B) and in three gradient profiles (*i.e.* non-linear, linear, and flat) of a microfluidic gradient device (C, D and E). MUC5AC-positive cells were indicated by brown color. Scale bars are 200 μm.

The gradient profiles of the microfluidic device were characterized using FITC-dextran (Fig. 1B). We observed a variety of gradient profiles across different regions of the microfluidic device. These included a non-linear gradient at 4 mm (“a” in Fig. 1B), a linear gradient at 13 mm (“b” in Fig. 1B), and a flat gradient at 29 mm (“c” in Fig. 1B) distance from the merging point of the three inlets. Analysis of the gradient profiles showed that the non-linear gradient profile ranged from 0 to 100% FITC-dextran, whereas the linear and flat gradient profiles ranged from 26 to 84 and 49 to 70%, respectively. It indicated that the gradient profiles varied significantly due to molecular diffusion and the diffusion was increased with the channel length. The configuration of the winding-shaped microchannel might be considered analogous to the meandering structure of airways in the lung. Therefore, when cells lining a stretch of airway epithelium are placed under the influence of locally released growth factors or chemokines, the cells would individually experience different levels of these mediators depending on their location in the epithelium, leading to different outcomes in cellular processes, such as proliferation, survival, and gene expression. Furthermore, our microfluidic gradient device could mimic flow profiles generated in the symmetrical model of the junction of the human bronchial tree (Reynolds number, $Re = 50–1500$) where air is transported [37]. Therefore, this microfluidic device could be a powerful tool to investigate the behavior of lung epithelial cells exposed to various gradient profiles.

3.2 Proliferation of NCI-H292 cells in a microfluidic gradient device

We investigated the morphology and proliferation of NCI-H292 cells cultured for 36 h in the microfluidic gradient device and in the 6-well plates (Fig. 2). Phase contrast images showed that NCI-H292 cells cultured in 6-well plates with EGF were more polarized and elongated than those cultured without EGF (Fig. 2A). However, we did not find an apparent difference in cell morphology in the non-linear EGF gradient profile of the microfluidic device (Fig. 2B). This was probably due to the effect of fluidic flow, since the continuous fluidic flow in a microfluidic device might remove secreted molecules that were released by the lung epithelial cells in an autocrine and paracrine fashion. Thus, unlike the cells cultured with EGF in static 6-well plate, the cells exposed to non-linear EGF gradient profile in the microfluidic device could not support their growth. However, the average shear stress in our microfluidic device (800 μm in width × 35 mm in length × 100 μm in thickness) was negligible (1×10^{-4} dyne/cm^2) as compared with the shear stress of vascular endothelial cells experienced in blood [23, 38]. We also analyzed the proliferation of NCI-H292 cells cultured in a microfluidic device and in a 6-well plate (Fig. 3). Proliferation was determined as the fold increase; the number of cells cultured for 36 h was divided by the number of cells initially seeded. The fold increases of NCI-H292 cells in 6-well plates without and with EGF were 2.1 ± 0.3 and 2.9 ± 0.3, respectively ($^*p < 0.05$) (Fig. 3A), indicating the stimulatory effect of EGF on the proliferation of lung epithelial cells. For the microfluidic gradient device, the fold increases in non-linear, linear, and flat gradient profiles were 1.8 ± 0.3, 2.0 ± 0.4, and 2.9 ± 0.9, respectively, indicating that the milder gradient profile enhanced cell growth (Fig. 3B). Although the proliferation of cells cultured with EGF in a 6-well plate was statistically higher than that of cells cultured without EGF ($^*p < 0.05$), we did not observe an apparent difference in cell proliferation in the three gradient profiles. The cell growth in non-linear and linear gradient profiles was lower than the growth of cells cultured with EGF in a 6-well plate.

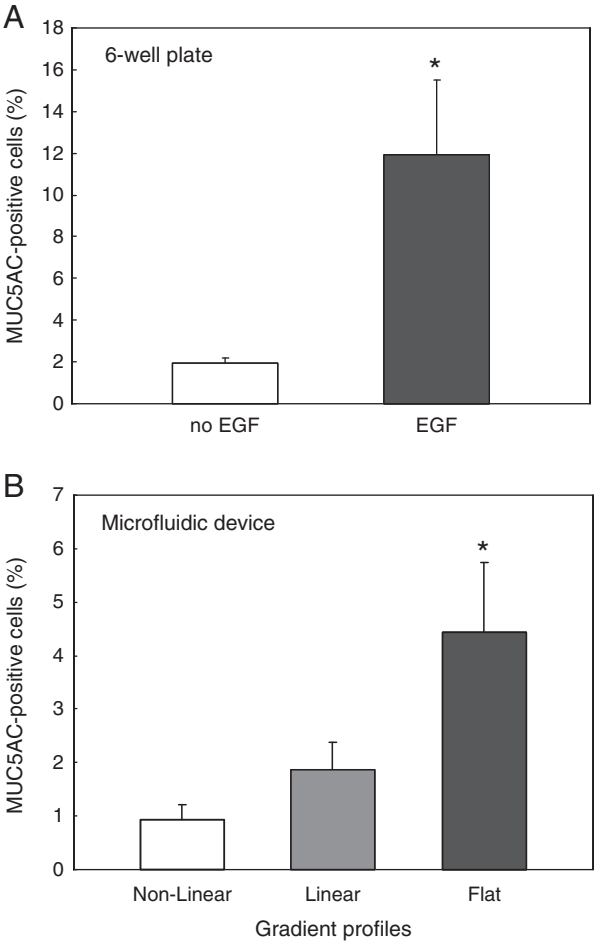

Figure 5. Quantitative analysis of MUC5AC expression. (A) Analysis of mucin expression of cells cultured in a 6-well plate. (B) Quantification of mucin-positive cells exposed to three EGF gradient profiles in a microfluidic gradient device. We quantified the percentage of MUC5AC-positive cells, showing that the number of MUC5AC-positive cells was divided by the total number of cell nuclei. Cells cultured in a 6-well plate with medium containing EGF showed high expression of MUC5AC ($^*p < 0.05$). Cells exposed to the flat gradient profile showed higher mucin expression as compared with the non-linear gradient profile ($^*p < 0.05$). All quantifications were performed three times at each gradient profile.

3.3 Mucin expression of NCI-H292 cells in a microfluidic gradient device

We examined the effect of the gradient profiles on MUC5AC expression. To enhance mucin expression, NCI-H292 cells were starved for 5 h in serum-free medium followed by culture with medium containing 2% FBS for 36 h. The MUC5AC expression level, measured by immunocytochemistry analysis, varied greatly among the patterns of gradient profiles, with the rank order of flat gradient > linear gradient > non-linear gradient (Fig. 4). Quantitative analysis showed that the percentage of MUC5AC-positive cells was $4.4 \pm 1.3\%$ in the flat gradient region, $1.9 \pm 0.5\%$ in the linear gradient profile, and was negligible in the non-linear gradient region ($0.9 \pm 0.3\%$) (Fig. 5). Thus, MUC5AC expression in the flat gradient was 4.8-fold higher than in the non-linear gradient ($^{*}p < 0.05$). However, it was noted that MUC5AC expression in response to flat EGF gradient profile was lower than that in 6-well plates with EGF ($11.9 \pm 3.6\%$).

We further analyzed mucin expression with respect to the distance from the merging point of the three inlets (Fig. 6A). The number of MUC5AC-positive cells was increased with the microchannel length (Fig. 6B). Mucin expression was low in the non-linear gradient region and then abruptly increased as the effect of the non-linear gradient faded, and steadily increased throughout the linear and flat gradient regions. Interestingly, the cells cultured within 25–35 mm distance from the merging point of the three inlets, where their cellular responses were dictated by the flat gradient profile, showed the highest percentage of MUC5AC-positive cells (> 3.8%) among the three gradient profiles. Therefore, we demonstrated that EGF gradient profiles enabled the control of mucin expression. Moreover, since EGF concentrations in the flat and linear gradient profiles ranged from 4.9 to 7.0 ng/mL and 2.6 to 8.4 ng/mL, most cells could be exposed to a similar range of EGF concentration in these two gradient profiles. Although they would be expected to have a similar cellular response, their MUC5AC expression was significantly different. The reason for the differential mucin expression is not clear. One possibility lies in the fact that MUC5AC expression might be related to cell proliferation. Unlike non-protein inducers, such as retinoic acid and butyric acid [39], that elicit differentiation of a wide range cell types by transactivating a particular set of genes without stimulation of cell proliferation, EGF not only induces gene transcription but also stimulates cell proliferation. Activation of mitogen-activated protein kinases is required for both the proliferation of NCI-H292 cells and the MUC5AC expression [40]. During the inflammatory and remodeling processes in the diseased lung, the two events occur simultaneously, leading to MUC5AC expression and hyperplasia of goblet cells. Therefore, culture of epithelial cells in the microfluidic gradient device might allow us to faithfully duplicate these cellular processes *in vivo* in a concerted fashion.

As a result, we demonstrated that the mucin expression of lung epithelial cells was significantly dependent on the EGF gradient profiles. Epithelial cells can yield different responses to a wide variety of normal and pathological mediators depending on their location in a stretch of epithelium. Therefore, the microfluidic gradient device provides a plausible tool to predict behaviors of the lung epithelial cells in response to growth factors.

4 Concluding remarks

We developed a microfluidic gradient device for regulating the mucin expression of NCI-H292 lung epithelial cells. A microfluidic device enables the generation of non-linear, linear, and flat EGF gradient profiles in a temporal and spatial manner. We demonstrated the effect of the gradient profiles on mucin expression of lung epithelial cells. Lung epithelial cells exposed to flat EGF gradient profiles exhibited high expression of the mucin gene as compared with cells exposed to non-linear and linear gradient profiles. Therefore, this microfluidic gradient device could be a powerful tool for controlling the function of lung epithelial cells and understanding cell biology.

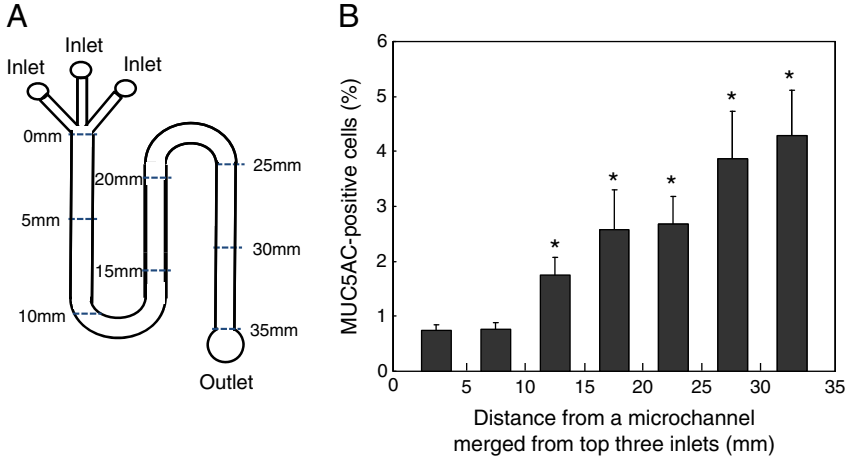

Figure 6. Quantification of MUC5AC-positive cells along the microchannel length. (A) Schematic diagram of the winding-shaped microchannel with respect to the distance from the merging point of the three inlets. (B) The percentage of MUC5AC-expressing cells was determined with respect to the distance along the microchannel. Asterisks indicate statistical significance as compared with the percentage of MUC5AC-positive cells at a 0–5 mm distance ($^{*}p < 0.05$).

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The authors have declared no conflict of interest.

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