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Enrichment of cancer-initiating cells from colon cancer cells through porous polymeric membranes by a membrane filtration method

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Cancer-initiating cells (CICs) or cancer stem cells (CSCs) are primarily responsible for tumor initiation, growth, and metastasis and represent a few percent of the total tumor cell population. We designed a membrane filtration protocol to enrich CICs (CSCs) from the LoVo colon cancer cell line via nylon mesh filter membranes with 11 and 20 μm pore sizes and poly(lactide-co-glycolic acid)/silk screen (PLGA/silk screen) porous membranes (pore sizes of 20-30 μm). The colon cancer cell solution was filtered through the membranes to obtain a permeate solution. Subsequently, the cell culture medium was filtered through the membranes to collect the recovery solution where the cells attached to the membranes were rinsed off into the recovery solution. Then, the membranes were cultivated in the cultivation medium to collect the migrated cells from the membranes. The cells migrated from any membrane had higher expression of the CSC surface markers CD44 and CD133, had higher colony formation levels, and produced more carcinoembryonic antigen (CEA) than the colon cancer cells cultivated on conventional tissue culture plates (control). We established a method to enrich the CICs (CSCs) of colon cancer cells from migrated cells through porous polymeric membranes by the membrane filtration protocol developed in this study.

Introduction

Cancer-initiating cells (CICs) or cancer stem cells (CSCs) generally comprise a few percent of the total tumor cell population.[1,2] This cell population is thought to be mainly responsible for tumor initiation, metastasis and growth. However, it is difficult to identify and distinguish CICs or CSCs from ordinal tumor cells. Several markers are reported to be CSC or CIC markers. For example, CD166,[3] CD133,[4-10] CD44,[5,7,10,11], CD24,[9,10] ALDH-1,[12-14] Musashi-1,[15] and Lgr5,[16,17] have been reported as colon CICs or CSCs. CD133 is the most famous marker of colon CICs or CSCs[4-10]; however, CD133 is also expressed on hematopoietic stem cells, and several researchers disagree on the use of CD133 as a marker of colon CICs or CSCs.[18,19] Currently, no reliable surface marker for colon CICs and CSCs is available. Therefore, it is not useful to isolate CICs or CSCs using separation methods, such as the magnetic-activated cell sorting (MACS) and fluorescence-activated cell sorting (FACS) methods, which rely on surface markers of CICs or CSCs. CICs or CSCs are defined as cells within tumors with capabilities of self-renewal, differentiation and tumorigenicity when transplanted into an animal host.[20] However, for the identification of cells as CICs or CSCs, we should perform many more mouse and rat experiments. Therefore, colony-forming or sphere-forming assays are typically used for the identification of CICs or CSCs instead of animal experiments, and colony-forming or sphere-forming assays help assess the ability of tumor-derived cells or CSCs from cell lines to propagate in vitro.[2,21,22]

The membrane filtration protocol was originally developed for stem cell separation by our group.[23-28] In our previous

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studies, selection of the membrane pore size and materials allowed the successful purification of hematopoietic stem cells (HSCs) and adipose-derived stem cells (ADCs), but not the CICs or CSCs intended to be isolated in this study, via membranes using a filtration and/or migration method.[23-28] In our previous research, we prepared poly(lactide-co-glycolic acid)/silk screen (PLGA/silk screen) membranes with pore size (r) = 24 μm, and human ADCs were successively isolated from primary human fat tissue solution into the permeate solution via PLGA/silk screen membranes by a membrane filtration protocol.[23] When a primary fat tissue solution or human ADCs were permeated through nylon mesh filter (NYM) membranes coated with recombinant vitronectin (r =11 or 20 μm), human ADCs were preferentially purified in the recovery solution or in the migrated cells from the NYM membranes.[25,28] On the other hand, porous polyurethane membranes with surface modification (r=5-12 μm) were successfully used for preferential isolation of HSCs from umbilical cord blood and peripheral blood.[26,27] Therefore, optimal materials and the optimal pore size of porous polymeric membranes should be selected for the isolation of stem cells depending on the characteristics of each specific stem cell.

In this study, we will isolate CICs or CSCs from a colon cancer cell line using the membrane filtration protocol via PLGA/silk screen membranes prepared with different PLGA solutions as well as NYM membranes with pore sizes of 11-29 μm. We selected LoVo cells to use as the colon cancer cell line in this study. This is because we are accustomed to use LoVo cells as a colon cancer cell line in several research of our different projects.[29,30] The purification efficiency of CICs (CSCs) was evaluated from (a) surface marker expression of CD133 and CD44, (b) colony forming assays and (c) production of the colon cancer cell marker protein, carcinoembryonic antigen (CEA).

We expect to develop an isolation method for CICs or CSCs from colon cancer cells to establish a patient-specific colon cancer cell line from a patient's primary colon cancer tissue by a membrane filtration protocol in the future, which should be beneficial for patient-specific cancer therapy and cancer cell assays.

Experimental

Materials

The chemicals and materials used in this project are listed in the ESI,† Table S1. The other biomaterials utilized in this project were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of PLGA/silk screen membranes

PLGA/silk screen membranes were made by a freeze-extraction protocol.[23,31-35] The schematic preparation procedures are described in Fig. 1A. PLGA was dissolved in DMSO (*N,N*-dimethylformamide) to prepare a 3, 5, and 10 wt% PLGA solution.[36-41] The PLGA solution was warmed at 79-81 °C for 30 min until a homogeneous PLGA solution was generated and was subsequently cooled to 25 °C. The silk screen mesh (170 mesh size) with circle dimensions (3.4 cm diameter) was injected into glass petri dishes (3.5 cm diameter), which were treated with high-vacuum grease to avoid membrane sticking. Then, 3 mL of the PLGA/DMSO solution was added to the glass dishes, including the silk screen mesh, and frozen at -21 °C for one day. The frozen PLGA/DMSO solution was immersed in 75% ethanol at -20 °C for 4 days, and 75% ethanol was exchanged twice a day to remove the DMSO solvent to form a porous structure (PLGA/silk screen membranes). Subsequently, the PLGA/silk screen membranes were placed in a fume hood at room temperature for 2 days and then dried in a vacuum oven at 30 °C for 24 hours to remove residual ethanol. PLGA/silk screen membranes prepared with 3%, 5%, and 10% PLGA solution were denoted as P-3/silk, P-5/silk, and P-10/silk membranes, respectively. Before the usage of the PLGA/silk screen membranes by the membrane filtration protocol, the membranes were sterilized by exposure to UV light for 24 hours.

Culture of colon cancer cell lines

Colon cancer cell lines (LoVo cells) were cultivated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 incubator and passaged using the conventional method.[29]

Isolation of CICs (CSCs) by the membrane filtration and migration protocol

The human colon cancer (LoVo) cell solution was permeated via PLGA/silk screen membranes or NYM membranes with pore sizes of 11 μm (NYM-11) and 20 μm (NYM-20) or P-3/silk, P-5/silk and P-10/silk porous membranes utilizing a batch-type membrane filter (C161, Millipore Corp.) (Fig. 1B). The membrane holders and filtration protocols utilized in this investigation were the same as those reported previously (Fig. 1B).[23-25, 42,43] The colon cancer cell solution with a total number of $1x10^6$ cells in 10 mL was permeated through the membranes at 25 °C with a permeation rate of 1.0 mL/min. The number of colon cancer cells in the feed solution and the permeate solution (N_f and N_p, respectively) was counted by utilizing a flow cytometry apparatus (BD Accuri™ C6, BD Biosciences). After the permeation of the colon cancer cell solution, the membrane holder was inverted, and DMEM supplemented with 10% FBS (recovery solution) was filtered through the membranes at a permeation rate of 1.0 mL/min at 25 °C. This step was performed to obtain the cells that had adhered to the membranes in the recovery solution (Fig. 1B). The total colon cancer cell number in the recovery solution (N_r) was counted using flow cytometry.

The permeation rate was investigated utilizing Eq. 1:

$$\text{Permeation rate (\%)} = (N_p/N_f) \times 100, \quad (1)$$

The recovery rate was evaluated utilizing Eq. 2:

$$\text{Recovery rate (\%)} = (N_r/N_f) \times 100, \quad (2)$$

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and the residual rate was estimated from the following equation:

Residual rate (%) = 100 (%) – [Recovery rate (%) + Permeation rate (%)]. (3)

After permeation of the recovery solution (wash solution), the membranes were removed from the filter holder and inserted into cell cultivation plates with DMEM supplemented with 10% FBS. The residual cells that adhered to the membranes migrated away from the membranes into the cell cultivation dishes when the membrane filters were cultivated for 30 days at 37 °C in a 5% CO_2 incubator. The total number of migrated cells was counted using flow cytometry.

The expression of CSC markers (CD133 and CD44) on the colon cancer cells in the feed, permeation and recovery solutions as well as on the migrated cells were investigated utilizing flow cytometry after staining with 7-AAD.

Colony forming assay

The soft agarose colony formation assay is a method for characterizing the anchorage-independent growth capability of cells in vitro,[22] which can be used to identify CSCs in vitro.[11,22,44-47] The procedure was followed according to previous publications,[22,48] with some modification as follows: (1) 2-hydroxyethyl agarose was dissolved in ultrapure water to prepare 3 wt% of 2-hydroxyethyl agarose solution, and subsequently, the agarose solution was autoclaved and stored at room temperature. (2) A 0.6 wt% agarose solution was prepared by diluting a 3 wt% agarose solution with ultrapure water at 45 °C, and 2 mL of the agarose solution at 45 °C was inserted into each well of 6-well plates. The plates were incubated on a flat surface at 4 °C for 1 hour to allow the mixture to solidify, and the agarose gel was ready for use. (3) The 0.6 wt% agarose solution was also prepared by mixing 2 mL of the 3% agarose solution with 8 mL of warm DMEM supplemented with FBS. Then, the cells were mixed with 0.6% agarose solution in a 1:1 dilution. Subsequently, 1 mL of the cell-agarose mixture with $2x10^4$ cells was added on the surface of agarose gel in the 6-well plate. The 6-well plate was placed in a 4 °C refrigerator for 15 min to allow the cell-agarose mixture layer to solidify. After solidification, the cell-agarose plate was incubated in a 37 °C incubator for 3 weeks where 100 µL of the culture medium was added twice a week to prevent desiccation. The colony appearance was investigated and imaged under a microscope 3 weeks after inoculation of the cells. Only colonies with diameters > 500 µm were identified as colonies, and the number of colonies was counted.

CEA production assay

The production of CEA by colon cancer cells was evaluated using an enzyme-linked immunosorbent assay (ELISA) kit[49] after the cells were permeated through NYM and PLGA/silk screen membranes. The cells in the permeate and recovery solutions as well as the migrated cells were cultivated on tissue culture polystyrene (TPS) dishes for 7 days. Subsequently, the cells

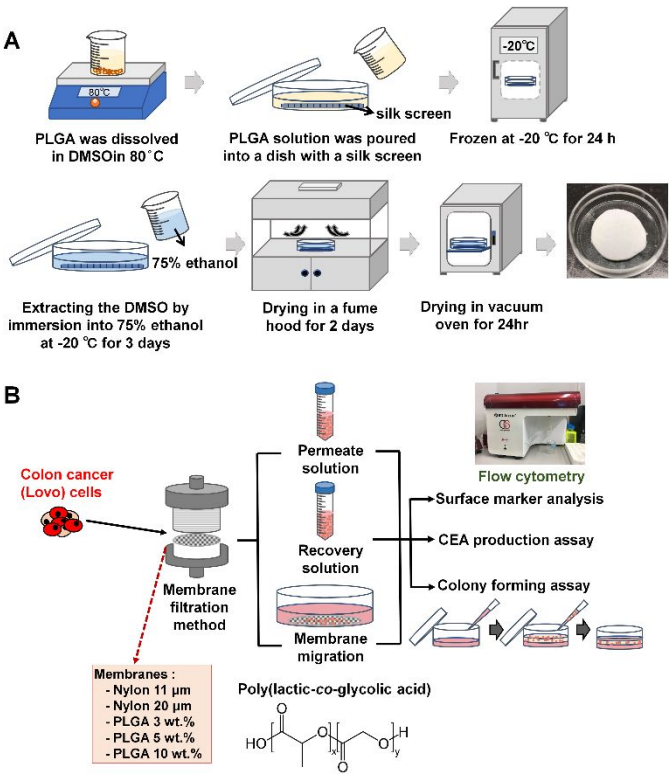

Fig. 1 Preparation of PLGA/silk screen membranes and filtration of colon cancer LoVo cells for isolation of CSCs or CICs. (A) Preparation method of the PLGA/silk screen membranes by the freeze-extraction protocol. (B) Membrane filtration and migration method to isolate CSCs or CICs of colon cancer LoVo cells. Colon cancer cell solution was filtered through the membranes to obtain permeate solution. Subsequently, the cell culture medium was filtered through the membranes to obtain the recovery solution. Then, the membranes were cultured in cell culture medium for 30 days to obtain the migrated cells. These cells were evaluated for CSC surface marker expression using flow cytometry, colony forming assays and CEA production assays.

were detached using trypsin-EDTA solution and centrifuged at 405 x *g* for 7 min to collect the cell pellet. The cell pellets were inserted into a 2 mL Eppendorf tube with 200 µl of DMEM, and the cells in the tube were lysed by freezing in liquid N2 for 30 sec and by melting in a 37 °C warm bath for 1 min. This freezing-melting process was repeated three times. After centrifugation at 405 x *g* for 7 min, the supernatant was collected, and the concentration of the supernatant was evaluated for the CEA production rate per cell. The CEA production rate was calculated from the following equation:

$$\text{CEA production rate (ng/10}^6\text{ cells)} = C*V*10^6 / Z \quad (4)$$

where C is the CEA concentration of the supernatant produced from LoVo cells, V is the volume of the medium (200 µL), and Z is the cell number.

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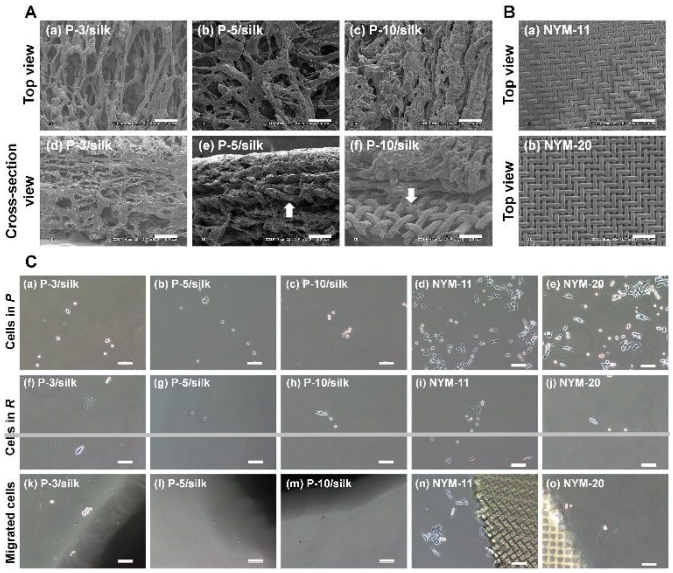

Fig. 2 Membrane structures and morphologies of colon cancer cells after filtration and migration from the membranes. (A) Morphologies of P-3/silk (a, d), P-5/silk (b, e), and P-10/silk (c, f) membranes from the top views (a-c) and cross-sectional views (d-f) analyzed using SEM. The scale bar indicates 200 μm. (B) Morphologies of NYM-11 (a) and NYM-20 (b) from the top views. The scale bar indicates 200 μm. (C) Colon cancer LoVo cell morphologies in permeate solution (*P*) (a-e), recovery solution (*R*) (f-j) and migrated cell (k-o) morphologies through the P-3/silk (a, f, k), P-5/silk (b, g, l), P-10/silk (c, h, m), NYM-11 (d, i, n) and NYM-20 (e, j, o) membranes. The scale bar indicates 100 μm.

Statistical analysis

All of the quantitative results were obtained from three samples. The data are expressed as the mean ± SD (standard deviation). Statistical analyses were performed using an unpaired Student's *t*-test in Excel (Microsoft Corporation). Probability values (*p*) less than 0.05 were considered statistically significant.

Results

Membrane characterization for the membrane filtration protocol

The NYM-11, NYM-20, P-3/silk, P-5/silk and P-10/silk membranes were characterized using scanning electron microscopy (SEM) in this study before the permeation of colon cancer cells through the membranes. Top views and cross-sectional views of the P-3/silk, P-5/silk and P-10/silk membranes as well as top views of the NYM-11 and NYM-20 membranes are shown in Figs. 2A and 2B. The P-3/silk, P-5/silk and P-10/silk membranes showed complicated morphologies with high tortuosity, which originated from pore generation by microphase separation, whereas the NYM-11 and NYM-20 membranes showed regular mesh morphologies because of the woven fabric structure. The average pore size of each membrane was evaluated by SEM using the ImageJ system (https://imagej.nih.gov/ij/) and is summarized in Table 1. The pore size of the membranes was evaluated to be between 11-29 μm. The pore sizes of NYM-11 and NYM-20 were evaluated to be 11.2 μm and 20.7 μm, respectively. The pore size of the P-10/silk membrane (22.9 μm), which was made of the highest concentration of PLGA in this study, was smaller than those of the P-3/silk (28.5 μm) and P-5/silk (27.5 μm) membranes. Because the size of colon cancer cells is approximately 10 μm, the pore size of the membranes used in this study is expected to be optimal, not too small and not too large for cell permeation through the membranes.

Table 1 Characterization of membranes used for membrane filtration and migration methods.

	Membranes				
	NYM-11	NYM-20	P-10/silk	P-5/silk	P-3/silk
Average pore size (μm)	11.2±2.1	20.7±1.3	22.9+2.4	27.5±2.0	28.5+2.7
Average thickness (μm)	5.7±0.6	5.8±0.3	141±13.5	92.7±2.3	83.0±2.6

Permeation of colon cancer cells through NYM and PLGA/silk screen membranes by the membrane filtration protocol

Colon cancer (LoVo) cells were permeated though the NYM-11, NYM-20, P-3/silk, P-5/silk and P-10/silk membranes by the membrane filtration and migration protocol. The morphologies of LoVo cells in the permeate solution and recovery solution and migrated cells were investigated under phase inverted microscopy, and the results are displayed in Fig. 2C. Only a few cells were observed in the recovery solution and only a few had migrated, whereas more cells were observed in the permeate solution than in the recovery solution. No significant difference was observed in the colon cancer cell morphology, and the cancer cells had permeated through any membrane along with the cells in the permeate and recovery solutions as well as the migrated cells.

The number of LoVo cells in the permeate and recovery solutions as well as the migrated cells were counted using flow cytometry, and the permeation rate, recovery rate and residual rate were calculated from these cell numbers using Eqs. 1-3. Fig. 3 shows the permeation rate, recovery rate and residual rate of LoVo cells by the membrane filtration protocol through the NYM (NYM-11 and NYM20) membranes and PLGA/silk screen (P-3/silk, P-5/silk and P-10/silk) porous membranes. The permeation rate through all of the NYM and PLGA/silk screen membranes investigated in this study was approximately 80% via any membrane (p>0.05). The recovery rate was found to be approximately 10% through any of the NYM and PLGA/silk screen membranes investigated in this study (p>0.05). Furthermore, the residual rate was found to be 6-15% through any of the NYM and PLGA/silk screen membranes investigated in this study. The residual rate of cells on the P-10/silk

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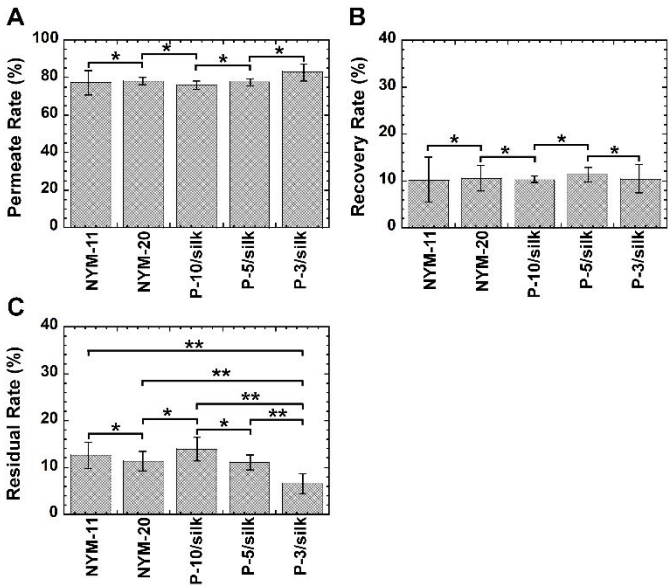

Fig. 3 Filtration characteristics after permeation of colon cancer LoVo cells through the NYM and PLGA/silk membranes. (A) Permeation rate of colon cancer cells through the NYM-11, NYM-20, P-10/silk, P-5/silk and P-3/silk membranes. (B) Recovery rate of colon cancer cells through the NYM-11, NYM-20, P-10/silk, P-5/silk and P-3/silk membranes. (C) Residual rate of colon cancer cells through the NYM-11, NYM-20, P-10/silk, P-5/silk and P-3/silk membranes. * p>0.05, ** p<0.05.

membranes was slightly higher than that on the P-3/silk membranes (p<0.05). This can be explained by the smaller pore size of the P-10/silk membranes than of the P-3/silk membranes. However, there was no significant difference in the permeation rate, recovery rate and residual rate for any membrane used in this study except the residual rate in P-3/silk membranes.

CSC marker expression on colon cancer cells permeated through the NYM and PLGA/silk screen membranes by the membrane filtration protocol

One of the indexes of whether CSCs (or CICs) are enriched or reduced after permeation through the membranes is an evaluation of the surface marker expression of CSCs (or CICs) by flow cytometry. In this study, we selected CD44[5,7,10] and CD133[4-10] expression on the cells to evaluate the enhancement or reduction in CSCs in colon cancer cells after permeation through the NYM and PLGA/silk screen membranes. ESI,† Figs. S1 and S2 show flow cytometry diagrams of CSC marker (CD44 and CD133, respectively) expression on LoVo cells in the permeate solution and recovery solution as well as on the migrated cells by the membrane filtration protocol through NYM membranes and PLGA/silk screen membranes where the migrated cells were collected after culture of the membranes in the culture medium for one month. The colon cancer cells in the permeate and recovery solutions as well as migrated cells were found to show CD44 and CD133 expression. LoVo cells cultivated on TPS dishes, which were not permeated through the membranes, showed approximately 60% CD44 and 70% CD133 expression.

The CD44 and CD133 expression on LoVo cells under each condition is summarized in Fig. 4. For CD44 expression, the cells in the permeate solution showed approximately the same expression of CD44 as the cells cultured on TPS dishes (p>0.05), except for the cells in the permeate solution through the P-10/silk membranes. The cells in the recovery solution also showed approximately the same expression of CD44 as the cells cultured on TPS dishes (p>0.05). The cells migrated through the NYM-11, NYM-20 and P-3/silk membranes expressed higher expression of CD44, whereas the cells migrated through the P-10/silk and P-5/silk membranes showed approximately the same expression of CD44 as the cells cultured on TPS dishes (p>0.05).

For CD133 expression, the cells in the permeate solution through the NYM-11, P-10/silk, P-5/silk, and P-3/silk membranes expressed higher expression of CD133 than the cells cultured on TPS dishes (p<0.05). The cells in the recovery solution were found to show approximately the same expression of CD133 as the cells cultured on TPS dishes (p>0.05). The cells migrated through all membranes studied in this study, the NYM-11, NYM-20, P-10/silk, P-5/silk and P-3/silk membranes, had higher expression of CD133 than the other cells (p<0.05). The cells migrated through the NYM-11, NYM-20 and P-3/silk membranes showed higher expression of both CSC markers, CD44 and CD133, than the cells cultured on TPS dishes (p<0.05).

Colony forming unit assay of colon cancer cells permeated through the NYM and PLGA/silk membranes by the membrane filtration protocol

Several researchers have reported that the high colony forming ability of cancer cells is directly related to the tumorigenicity of the cells.[11,21,22,46] Therefore, we investigated the colony forming assay of LoVo cells before and after permeation through the NYM and PLGA/silk screen membranes. Fig. 5A-5D shows colony forming pictures on agar gels by LoVo cells in the permeate solution (Fig. 5A) and recovery solution (Fig. 5B) as well as migrated cells (Fig. 5C) after permeation through the NYM and PLGA/silk membranes. A colony forming picture of the LoVo cells cultured on TPS dishes (control) is also shown in Fig. 5D. The number of colonies was counted using the ImageJ system (https://imagej.nih.gov/ij/) under each condition and is summarized in Fig. 6. The colony forming numbers of LoVo cells in the permeate solution and recovery solution were found to be approximately the same or less than that of the cells cultured on TPS dishes (control) except for the cells in recovery solution through the P-3/silk membranes.

The colony forming rate of cells migrated through the membranes was found to be higher than that of the cells cultured on TPS dishes (control) (p<0.5), except for the cells migrated through the NYM-20 membranes, where the colony forming rate of the cells migrated through the NYM-20 membranes was found to be approximately the same as that of the cells cultivated on TPS dishes (p>0.05).

It was found that cells with high tumorigenicity, which may be CSCs, could be enriched in the cells migrated through the

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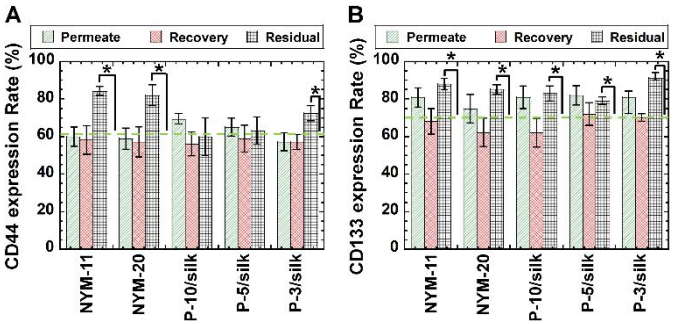

Fig. 4 CSC (CIC) surface marker expression on colon cancer LoVo cells analyzed by flow cytometry after permeation through the NYM and PLGA/silk membranes. (A) CD44 expression on the cells in the permeate solution, recovery solution and cells migrated through NYM-11, NYM-20, P-10/silk, P-5/silk and P-3/silk membranes. (B) CD133 expression on the cells in the permeate solution, recovery solution and cells migrated through NYM-11, NYM-20, P-10/silk, P-5/silk and P-3/silk membranes. The dotted line expresses CD44 (A) and CD133 (B) expression in LoVo cells cultured on TPS dishes. * p<0.05.

NYM-11 and PLGA/silk screen membranes from the colony forming assay in this study. It should be mentioned that the cells migrated through the NY-11 and P-3/silk membranes showed higher expression of the CSC surface markers CD44 and CD133 in this experiment than the cells cultivated on TPS dishes, which was consistent with the high numbers of colonies forming on agar gels by the cells migrated through the NYM-11 and P-3/silk membranes.

CEA production by LoVo cells permeated through the NYM and PLGA/silk screen membranes by the membrane filtration protocol

CEA production is one of the characteristics of colon cancer cells. In a previous experiment, the cells migrated through the NYM-11 and P-3/silk membranes showed high CSC surface marker expression of CD44 and CD133 and high numbers of colony formation on agar gels. Therefore, CEA production by LoVo cells in the permeate solution and recovery solution as well as migrated cells after permeation though the NYM-11 and P-3/silk membranes by the membrane filtration protocol was evaluated, and the results are shown in Fig. 7, where the cells were lysed by a freezing-melting process and the CEA produced by the cells was extracted in the medium. CEA production was calculated from the total cell number and CEA concentration in the medium extracted from the cells using Eq. 4. LoVo cells in the permeate solution and recovery solution through P-3/silk membranes produced approximately the same amount of CEA as the cells cultured on TPS dishes (control), whereas the cells in the permeate solution and recovery solution through NYM-11 membranes as well as cells migrated through the NYM-11

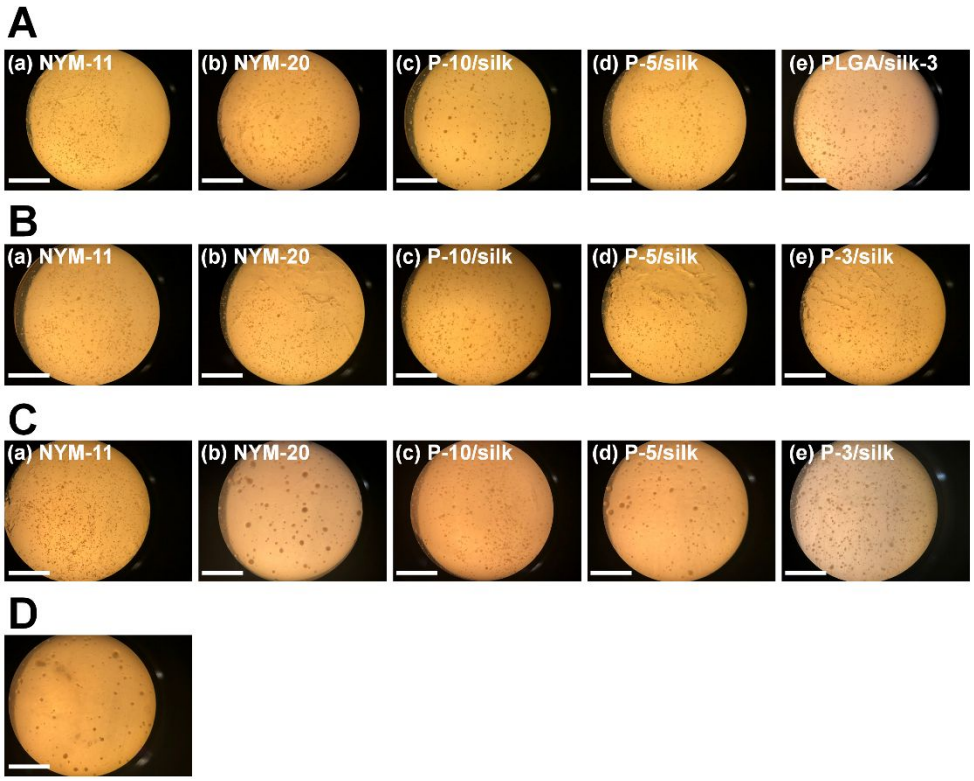

Fig. 5 Colony forming assay of colon cancer LoVo cells after permeation through the NYM and PLGA/silk membranes. (A) Colony forming pictures of colon cancer cells in the permeate solution through the NYM-11 (a), NYM-20 (b), P-10/silk (c), P-5/silk (d) and P-3/silk (e) membranes. The bar indicates 1 cm. (B) Colony forming pictures of colon cancer cells in the recovery solution through the NYM-11 (a), NYM-20 (b), P-10/silk (c), P-5/silk (d) and P-3/silk (e) membranes. The bar indicates 1 cm. (C) Colony forming pictures of cells migrated through the NYM-11 (a), NYM-20 (b), P-10/silk (c), P-5/silk (d) and P-3/silk (e) membranes. The bar indicates 1 cm. (D) Colony forming pictures of LoVo cells cultivated on TPS dishes. The bar indicates 1 cm.

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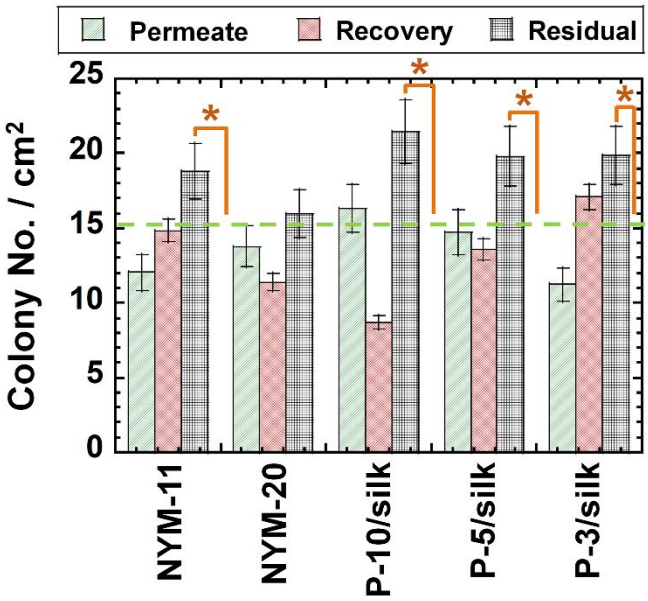

Fig. 6 The number of colony forming colon cancer cells in the permeate solution and recovery solution and the cells migrated through the NYM-11, NYM-20, P-10/silk, P-5/silk and P-3/silk membranes by colony forming assays. * $p< 0.05$.

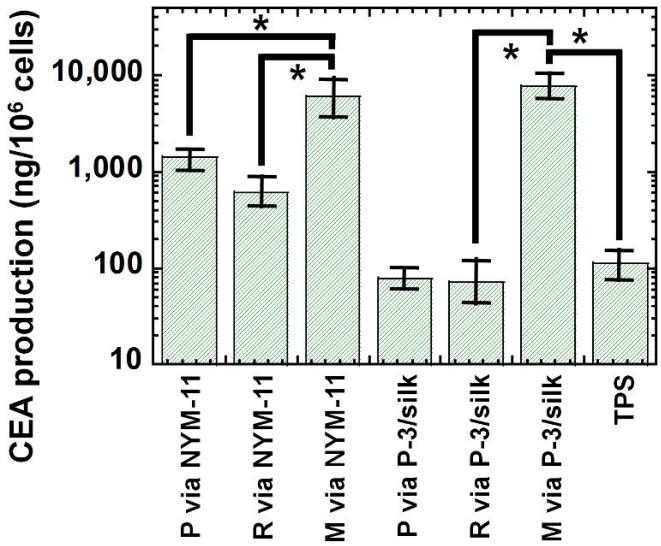

Fig. 7 CEA production of colon cancer LoVo cells after permeation through the NYM and PLGA/silk membranes. CEA production rate analyzed after 7 days of culture of colon cancer LoVo cells in the permeate (P) and recovery (R) solutions as well as cells migrated (M) through the NYM-11 and P-3/silk membranes. * $p<0.05$.

and P-3/silk membranes produced much higher CEA levels than the cells cultured on TPS dishes (control) (p <0.05). In particular, the cells migrated through both the NYM-11 and P-3/silk membranes produced higher amounts of CEA than the cells in the permeate solution and recovery solution through both the NYM and P-3/silk membranes and higher amounts than LoVo cells cultured on TPS dishes (p <0.05).

Discussion

The LoVo cells migrated through the NYM-11 and P-3/silk membranes showed higher levels of the CSC surface markers CD44 and CD133 as well as higher colony forming numbers and higher CEA production than LoVo cells cultured on TPS dishes. The migrated cells are regarded as the most adherent and sticky cells on the membranes because weaker adherent cells cannot adhere to the membranes during permeation of LoVo cell solution and can be collected in permeate solution. Subsequently, the middle adherent cells on the membranes, which adhered on the membranes with weak interaction, can be rinsed out into the recovery solution when the recovery solution (culture medium) is permeated through the membranes. The highly adherent cells on the membranes, which could not be washed out with the recovery solution, were cultivated in the culture medium for one month. The cells with high mobility migrated out from the membranes and could be collected as migrated cells after one month of cell culture on the membranes. Therefore, cells with high adherence and high mobility characteristics can be isolated by the membrane filtration and migration protocol, and the cells seem to have CSC or CIC characteristics based on the high CSC surface marker expression, high colony forming potential and high CEA production.

Some cell sorting (purification or isolation) protocols have been developed to isolate the specific characteristics of the cells. These protocols are (a) the selection of genetically edited cells that express targeted genes along with antibiotic resistance genes from antibiotic treatment (e.g., zeocin, G418, neomycin, and puromycin),[50-55] (b) FACS[56-60] and (c) MACS.[61,62] In the FACS and MACS purification protocols, fluorescence (e.g., green fluorescence protein; GFP) by genetically edited targeted cells or antibodies binding to the targeted cells must be detected, which causes contamination by antibodies or genetically edited cells of the final products of isolated cells. In terms of protocol (a), genetic editing of the cells is considered to be inadequate for clinical use, although the targeted cells are easily isolated for use in research with high purity. A safe and less laborious cell sorting protocol to isolate the targeted cells, such as the membrane filtration and migration protocol, is needed, and this research verifies this method for the isolation of CSCs or CICs from a cancer cell line, LoVo cells.

Conclusions

We investigated whether CSCs or tumorigenic cells (CICs) of colon cancer cells (LoVo cells) could be isolated by the membrane filtration protocol in this study. The cells in the permeate solution and recovery solution showed no difference in the CSC surface marker expression of CD44 and similar or fewer colony forming cell numbers of the cells cultured on agar

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gels compared to those of the cells cultured on TPS dishes, which indicated that CSCs or CICs could not be isolated from the cells in the permeate solution and recovery solution through the NYM or PLGA/silk membranes in this research. However, compared to the cells cultured on TPS dishes, the cells migrated through the NYM membranes and PLGA/silk membranes expressed higher levels of the CSC surface markers CD44 and CD133 and showed higher numbers of colony forming cells, which indicated that CSCs or tumorigenic cells (CICs) could be isolated from the cells migrated through the NYM and PLGA/silk membranes in this study. Furthermore, the migrated cells showed higher production of CEA than the cells cultured on TPS dishes. Compared to conventional FACS and MACS, the membrane filtration and migration protocol should be valuable for cell sorting and isolation of targeted cells, such as CSCs (CICs), without the need for antibody binding or genetic editing of the cells.

Conflicts of interest

There are no conflicts to declare.

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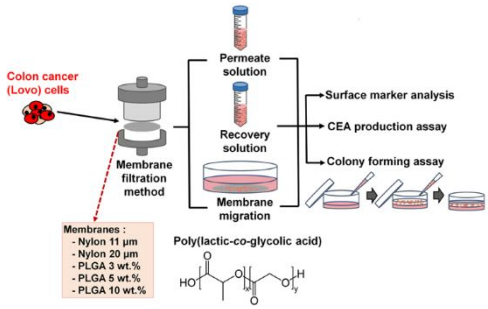

A method to enrich the cancer stem cells of colon cancer cells through porous polymeric membranes is developed.