Development of bioartificial renal tubule devices with lifespan-extended human renal proximal tubular epithelial cells

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Abstract
Background. The bioartificial renal tubule device is a cell therapy system for renal failure. The major obstacle in the development of the bioartificial renal tubule device is the obtainment of a large number of viable renal tubule cells to seed on the inner surface of hollow fibers. Although our previous studies had used a transformed cell line, they may be dangerous for clinical uses. Therefore, different approaches to amplify renal proximal tubular epithelial cells (RPTEC) in culture without oncogenes, vectors and carcinogens have been required.

Methods. The limitation of the replicative lifespan of human RPTEC, which is ~12 population doublings (PDs), was extended by invalidating messenger RNA of cell cycle-related genes with antisense oligonucleotide or small interfering RNA (siRNA).

Results. Periodic transfection of siRNA to a tumor suppressor p53 or a cyclin-dependent kinase inhibitor p16INK4A extended the lifespan by 33 and 63 PDs, respectively, in 3 months of culture. The siRNA-mediated lifespan extension was controllable because cell division ceased within 2 weeks after the transfection was discontinued. Expressions of γ-glutamyltransferase 1 and glucose transporter 1 were recovered in siRNA-transfected RPTEC cultured on porous membranes. Bioartificial renal tubule devices (0.8 m²) constructed with these cells showed reabsorption of water (122.3 ± 4.2 mL/30 min), sodium (18.1 ± 0.7 mEq/30 min) and glucose (121.7 ± 4.4 mg/30 min) after 1 week of circulation. Furthermore, β2-microglobulin and pentosidine were metabolized by RPTEC in mini-devices (65 cm²) within 48 h of circulation.

Conclusions. These approaches enabled us to yield a high enough number of RPTEC for construction of bioartificial renal tubule devices repeatedly. Lifespan-extended RPTEC could recover their specific characteristics by culturing on porous membranes, and bioartificial renal tubule devices constructed with these cells showed good performances of reabsorption and metabolism.

Summary: A large number of human renal tubular cells required for construction of the bioartificial renal tubule device were prepared by extending the lifespan of the primary cells by invalidating mRNA of cell cycle-related genes. Constructed bioartificial renal tubule devices with lifespan-extended cells showed good performances of in vitro examination of reabsorption and metabolism. Requiring no oncogenes, vectors or cell cloning, the RNAi-mediated lifespan extension can help advance tissue-replacement therapy as well as basic research.

Keywords: bioartificial renal tubule device; renal proximal tubular epithelial cells; replicative lifespan; RNA interference

Introduction

The bioartificial renal tubule device is a cell therapy system for renal failure (for review, see ref. [1, 2]). This system improves the present hemodialysis by adding abilities of renal tubular cells seeded on the inner surface of hollow fibers. Useful small molecules, such as water, electrolytes, glucose and amino acids, that have been discarded with uremic toxins in the present blood purification therapies, could be reabsorbed selectively and returned to the body by seeded cells. The device is also expected to perform metabolic and endocrine functions of tubular cells. Humes et al. [3] showed these functions by constructing a bioartificial renal tubule assist device with porcine renal proximal tubular cells seeded into the intraluminal spaces of hollow fibers. They also reported initial clinic results of a bioartificial kidney containing human renal proximal tubular epithelial cells (RPTEC) in ICU acute renal failure patients who displayed satisfactory outcomes [4, 5]. These reports encouraged the development and improvement of the bioartificial renal tubule device for clinical use.

The major obstacles in the development of the bioartificial renal tubule device are the obtainment of a large number of viable renal tubular cells and an adequate and even lining of the intraluminal surface of hollow fibers of modules with these cells in order to maintain cell functions over
a longer period of time. Since it is difficult to collect as many kidneys as needed to isolate a high enough number of cells at once, we have amplified human RPTEC in culture. However, because of a limitation of the amplification of the primary cultures of human RPTEC at ~12 population doublings (PDs), it has been difficult to supply a high enough number of RPTEC with constant quality for development of practical devices. Although RPTEC transformed by oncogenes can proliferate permanently, they are thought to be dangerous for use in medical devices. Furthermore, immortalized cells continue growing in hollow fibers, so that they make multilayers and block active transports [6]. One of the solutions for these problems is extension of the replicative lifespan of the primary cells.

Cells isolated from normal human tissues undergo a limited number of divisions in vitro and enter a nondividing state called replicative senescence [7, 8]. Senescence has been related to telomere shortening [9] and increases in the cyclin-dependent kinase inhibitors p16<sup>INK4a</sup> and p21<sup>CIP1</sup> [10], the components of the p53/p21<sup>CIP1</sup> and p16<sup>INK4a</sup>/pRB pathways that arrest the cell cycle progression. Inactivation of p53 and/or pRB by papillomavirus E6/E7 or SV40 large T-antigen or of p21<sup>CIP1</sup> allows proliferation beyond the normal limit [11–14]. Introduction of the telomerase has been shown to extend the replicative lifespan of human cells [15]. However, some types of cells, such as epithelial cells and keratinocytes, are refractory to lifespan extension by telomerase alone, even when required inactivating of the p16<sup>INK4a</sup>/pRB pathway [16–19].

Earlier studies on human RPTEC immortalization also employed human papillomavirus E6/E7 [20] or SV40 large T-antigen [21–23]. Recently, the human telomerase was transduced via a retrovector and successfully immortalized human RPTEC [24]. The sole report of reversibly immortalized RPTEC used the Cre-loxP system for inducible deletion of transduced telomerase and SV40 large T-antigen [25]. However, all these studies required introduction of oncogenes or promoters derived from virus into cells.

Here, we report a safe and effective means of extending the replicative lifespan of normal human RPTEC in vitro by suppressing gene expressions of cell cycle-related genes using antisense oligonucleotides (ASOs) [26, 27] or RNA interference (RNAi) [28, 29] and show good performances of the bioartificial tubule devices applied with lifespan-extended RPTEC.

Subjects and Methods

This study protocol was approved by the Institutional Review Board of Tokai University School of Medicine. Human materials were used under patients’ informed consent.

Cell culture

Human RPTEC were isolated by immunomagnetic separation [30] from kidneys removed for therapeutic purposes. Fifty thousand RPTEC were seeded in one well of six-well plates and cultured in Renal Epithelial Growth Medium (LONZA, Basel, Switzerland) at 37°C in 5% CO<sub>2</sub> in the atmosphere. Media were changed every 2 days. Subculture was repeated at ~2-week intervals. In some experiments, cells were cultured on porous membranes (Transwell® Permeable Supports 3450; Corning, NY).

For large-scale culture, 3–5 × 10<sup>6</sup> isolated RPTEC were plated in a 225-cm<sup>2</sup> flask (BD, Franklin Lakes, NJ). Approximately 1 week later, subconfluent cells were transfected with p16<sup>INK4a</sup> small interfering RNA (siRNA) and subcultured on the following day on two 632-cm<sup>2</sup> chambers of a large-scale culture system (Nunc<sup>TM</sup> Cell Factory; Nalgé Nunc, Rochester, NY). When subconfluent, cells were transfected with siRNA again and subcultured on one 6320-cm<sup>2</sup> chamber (10 layers of 632-cm<sup>2</sup> chambers) and two 632-cm<sup>2</sup> chambers. Cells were harvested from 6320-cm<sup>2</sup> chamber for construction of bioartificial renal tubule devices, and cells on two 632-cm<sup>2</sup> chambers were repeatedly suffered transfection and subcultured in the same manner.

PDs were determined using the numbers of cells seeded and those harvested on the following day: PD = log<sub>2</sub> (the number of harvested cells/the number of seeded cells).

Antisense oligonucleotides

Phosphorothioated ASOs to p53 (5′-CGGCTCTCCATGGCCAGT-3′), p16<sup>INK4a</sup> (5′-TGTGCTCTCCCGCGCCC-3′), RB (5′-GTGACGACAATCTTCTTACGG-3′) or p21<sup>CIP1</sup> (5′-AGGCCGTTTCGACATGGCG-3′) and a negative control oligonucleotide (5′-ACGTGACACGTCCGGAGA-3′) were obtained from TSUKUBA OLIGO SERVICE (Ushiku, Japan), dissolved in distilled water at 1 mM and stored at −20°C. The oligonucleotides were added to cultures to a final concentration of 10 μM. Media were changed every 2 days to media containing the control oligonucleotide or ASOs.

RNA interference

siRNAs to p53 (5′-CCAGUGUAACUUCAUGGGACGGA-3′) or p16<sup>INK4a</sup> (5′-AGGGCAUGUACCCGUCCGUAAGU-3′) and a negative control double-stranded RNA (dsRNA) (Medium GC) were obtained from Invitrogen (Stealth RNAi; CA), dissolved in distilled water at 20 μM and stored at −20°C. siRNAs were mixed with a transfection reagent (RNAiMax; Invitrogen) in Opti-MEM I medium (Invitrogen) and, after 20 min, media were changed at room temperature, added to cultures to a final concentration of 50 nM in media without antibiotics. On the following day, media were changed to one with antibiotics.

Quantitative real-time polymerase chain reaction

Real-time polymerase chain reaction (PCR) was performed on ABI PRISM7500 (Applied Biosystems, Carlsbad, CA) as described [31] using Taqman Gene Expression Assay kits (Applied Biosystems) for human cell-specific lifespan markers. Real-time polymerase chain reaction (PCR) was performed on ABI PRISM7500 (Applied Biosystems, Carlsbad, CA) as described [31] using Taqman Gene Expression Assay kits (Applied Biosystems) for human p16<sup>INK4a</sup> (Applied Biosystems) for human p16<sup>INK4a</sup>, p21<sup>CIP1</sup>, p53, or pRB, and human telomerase. Relative amounts of target messenger RNAs (mRNAs) were determined by qRT-PCR on ABI PRISM7500 (Applied Biosystems, Carlsbad, CA) as described [31] using Taqman Gene Expression Assay kits (Applied Biosystems) for human p16<sup>INK4a</sup>, p21<sup>CIP1</sup>, p53, or pRB, and human telomerase.

Assays of cell functions

Cells cultured on conventional plates or porous membranes were examined in functional assays of GGT1 and GLUT1. GGT1 activity was determined as reported by Wieser et al. [24] with minor changes. When confluent, substrate solution (1 mM γ-glutamyl-para-nitroanilide and 20 mM glycyl-glycyl-glycine in 60 mM Tris–HCl, pH 8.0) was added and incubated for 20 min at 37°C to determine GGT1 activity. Reaction was stopped by adding a half volume of 10% acetic acid, and para-nitroanilide release was observed by spectrophotometer at 405 nm.

GLUT1 activity as Na-independent 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amin0]-2-deoxy-o-glucose (2-NBDG) uptake was determined as reported by Yamada et al. [32] with minor changes. Culture medium was replaced with uptake buffer containing 137 mM choline chloride, 5.4 mM KCl, 2.8 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, and 1 mM KH<sub>2</sub>PO<sub>4</sub> and 800 μM of 2-NBDG was added to the culture. After incubation for 20 min at 37°C, uptake buffer was washed out and fluorescence of 2-NBGD was observed by fluorescence microscopy at 520–560 nm wavelength (excitation wavelength: 465–495 nm).

Preparation of bioartificial renal tubule devices

The ethylene vinyl alcohol (EVAL) hollow fiber modules (0.8 m<sup>2</sup>) were purchased from Asahi Kuraray Medical Co. Ltd. (Tokyo, Japan). After washing with phosphate-buffered saline (PBS), inner surface of hollow fibers were pre-coated with 10 μg/ml attachin (Bio999, Taipei, Taiwan) for 10 min. Seeding of RPTEC was repeated four times at intervals of 1 h each, accompanied with 90° rotation of the module. Total 3–7 × 10<sup>6</sup> cells were inoculated in a module. Culture medium was circulated at a rate of 20 mL/min on the outer compartment of the module. From 1 h after seeding, circulation on the inner compartment started at the same rate. Culture media were changed every day during closed circuit circulation perfusion for >2 weeks.

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Mini-modules of EVAL hollow fibers (65 cm²) were kindly supplied by Nipro (Osaka, Japan). Total $5 \times 10^5$ of RPTEC were inoculated in the same manner, and circulation was performed at a rate of 0.25 mL/min.

Scanning electron microscopy

Observation by scanning electron microscopy (SEM) was performed as described previously [6]. Briefly, cells in bioartificial renal tubule devices were washed with PBS, prefixed with 2.5% glutaraldehyde in PBS for 2 h at 4°C. After washing with PBS, the fibers were recovered from the module and sectioned into pieces. Samples were washed twice in PBS and re-fixed with 1% osmium tetroxide in PBS for 1 h at 4°C. Then, the samples were gradually dehydrated in ethanol solutions and tert-butyl alcohol and dried. After coating with gold in an ion sputter coater (JFC-110; JOEL, Tokyo, Japan), cell-attached hollow fibers were examined with a scanning electron microscope (JSM-840A; JOEL).

Evaluation of leakage and reabsorption

Leakage of creatinine and urea nitrogen and reabsorption of water, sodium and glucose through the hollow fibers were examined by single-pass perfusion. The basal medium containing 5.0 mg/dL creatinine and 20 mM urea was passed through the inner compartment of the device. Samples were taken from medium bottles, before and 30 min after perfusion, and media were weighed. Concentrations of creatinine, urea nitrogen, sodium and glucose were measured using standard laboratory assays and movements of substances across the hollow fibers were calculated as described previously [6].

Metabolism assays

β2-Microglobulin (Sigma, St. Louis, MO) was dissolved in Dulbecco’s modified Eagle’s medium (DMEM) culture medium containing 10% fetal calf serum (FCS) at a concentration of 5 mg/L. Ten milliliters of the solution was circulated in a closed circuit passing through the inner compartment of the mini-device, and DMEM culture medium with 10% FCS was filled in the outer compartment. Less than 1 mL of the medium was collected from the fluid at each time point. β2-Microglobulin concentrations of collected samples were measured using standard laboratory assay.

For pentosidine assays, uremic serum was collected from renal failure patients. Before circulation, uremic serum was 1:1 diluted with DMEM culture medium without serum. Circulation in the mini-device and sample collections were performed as in the case of β2-microglobulin. As controls, diluted uremic serum was circulated in the mini-device without cells, and samples were collected for measuring backgrounds. Samples were incubated with 6N HCl for 16 h at 110°C under nitrogen. After neutralization with 0.5N NaOH, samples were diluted with PBS and injected into high-performance liquid chromatography (model LC-10A; Shimadzu, Kyoto, Japan). Fluorescence at 385 nm was monitored [33].

Statistics

Data are presented as means ± SD. Student’s t-test was used to compare data. P-values for statistical significance are as indicated.

Results

Extension of the lifespan of human RPTEC with ASOs

To examine whether invalidation of mRNA of cell cycle–related genes extends the replicative lifespan of RPTEC, human RPTEC were cultured in the continued presence of ASOs to pRB, p53, p21Cip1 or p16INK4a (Figure 1). The control oligonucleotide had no effect. Nontransfected and control oligonucleotide-transfected cells ceased proliferation after ~12 PD. ASOs to RB, p53 or p21Cip1 extended the replicative lifespan by ~6–8 PDs and p16INK4a ASO by 10 PD before cells eventually ceased to divide. Results indicated effective extension of lifespan of RPTEC by invalidating mRNA of cell cycle–related genes.

Extension of the lifespan of human RPTEC by RNAi

The siRNA for p53 and p16INK4a were individually transfected into human RPTEC every 2 weeks. Periodic transfection with siRNAs to p53 or p16INK4a reduced the target mRNA levels to 10–20% of that in nontransfected cells (Figure 2). The control dsRNA had no effect. Nontransfected and control dsRNA-transfected cells ceased proliferation after ~12 PD. p53 or p16INK4a siRNA increased the replicative lifespan by 33 PD and 63 PD, respectively, in 3 months of culture before the cultures were terminated.

Effective duration of p16INK4a siRNA was examined by discontinuing the periodic transfection in a set of cells at various times points of culture (Figure 3). When the transfection was discontinued before 12 PD (by Day 46 in this experiment), cells ceased to divide after a total of ~12 PD. When the transfection was discontinued after 12 PD (on and after Day 69), cells ceased to divide within 2 weeks.

Functional characteristics of lifespan-extended RPTEC

As function markers of RPTEC, expressions and activities of GGT1 and GLUT1 were examined. The mRNAs of GGT1 and GLUT1 decreased in p16INK4a siRNA-transfected cells cultured on conventional plates (Figure 4, left panels). When cells were transferred to porous membranes, the mRNA expression recovered to comparable or higher levels as compared to those in cells of early PD on conventional plates. Addition of albumin to the outer side of the culture inserts further increased GLUT1 and
mRNA levels. The recovery of both GGT1 and GLUT1 were confirmed by their enzymatic activities (Figure 4, right panels).

**Large-scale culture of lifespan-extended RPTEC**

We also performed large-scale cultures of lifespan-extended RPTEC according to the protocol in Table 1. A large part of amplified cells were used for the construction of bioartificial renal tubule devices, and the remaining cells were reamplified after transfection with p16INK4a siRNA. This protocol enabled repeated yielding of large numbers of cells for long periods.

Table 2 shows a process of the representative large-scale culture that was initiated with $4.1 \times 10^6$ cells of isolated human RPTEC in a 225-cm$^2$ culture flask. After an overnight incubation, living cells ($3.9 \times 10^6$ cells) were subcultured in a new 225-cm$^2$ culture flask (Day 1). About 1 week later when cells were subconfluent, cells were transfected with p16INK4a siRNA and harvested on the following day, and $1.8 \times 10^7$ cells were plated onto two 632-cm$^2$ culture chambers of a large-scale culture system (Day 7). PD was 2.2 from the preceding subculture. On Day 14, cells transfected on the previous day were harvested, and $5.8 \times 10^7$ cells (1.7 PD from the preceding subculture) were plated onto 6320-cm$^2$ chambers and two 632-cm$^2$ chambers (Passage 3). On Day 21, cell yield from the 6320-cm$^2$ chamber was $5.5 \times 10^8$ cells (3.4 PD from the preceding subculture), which was used for construction of a bioartificial renal tubule device yielded $5.5 \times 10^7$ cells from two 632-cm$^2$ chambers were plated on a 6320-cm$^2$ chamber and two 632-cm$^2$ chambers after transfection on the previous day. Thereafter, cell yield at subculture ranged from $3.3 \times 10^8$ to $6.5 \times 10^9$ cells, PD ranged from 2.4 to 3.9 and 5.0 to 7.3 to 10 cells were used to expand the culture. By 70 days of culture, a total of $3.5 \times 10^9$ cells were harvested from the initially seeded $4.1 \times 10^6$ cells, and the total PD were 27.8.
Transport studies of bioartificial tubule devices constructed with lifespan-extended RPTEC

Bioartificial renal tubule devices (0.8 m²) were seeded with lifespan-extended RPTEC on the inner surface of hollow fibers. During >2 weeks of circulation of culture medium, leakage of creatinine and urea nitrogen and reabsorption of water, sodium and glucose were evaluated (Figure 5). Though the leakage of creatinine and urea nitrogen was ~30% in a few days from the start of circulation, it decreased gradually in a week and stabilized at ~15%. Contrary to leakages, reabsorptions increased during the first week of incubation and reached maximum levels after Day 7. Observation by SEM revealed that the inner surface of the hollow fibers was covered with confluent monolayer of living cells (Figure 6A). Microvilli were well developed on the apical surface of cells (Figure 6B). Averaged transport activities of water, sodium and glucose after the seventh day of circulation were 122.3 ± 4.2 mL/30 min, 18.1 ± 0.7 mEq/30 min and 121.7 ± 4.4 mg/30 min, respectively (n = 4).

Metabolism assays

Metabolic abilities of bioartificial renal tubule devices were examined by adding uremic toxins in the circulation. Figure 7 shows the metabolic decrease of β2-microglobulin and pentosidine in bioartificial renal tubule mini-devices (65 cm²) in the closed circuit. The decrease was detectable after 4 h of circulation, and >90% of uremic toxins added into the circulation were metabolized within 48 h.
Discussion

Uremic conditions such as serum accumulation of uremic toxins, malnutrition and impaired metabolism and deregulation of inflammatory response still remained in patients treated with current dialysis treatment as the renal replacement therapy, which results in poor prognosis in acute kidney injury (AKI) patients and severe dialysis-related complications in chronic dialysis patients. The insufficiency of current renal replacement therapy will be able to be improved in addition to treatment using the bioartificial renal tubule device. In order to prepare a high enough number of RPTEC for worldwide demand for treatments of AKI and chronic kidney disease (CKD) patients, we investigated the development of useful and safe methods of massive proliferation of RPTEC.

We employed two invalidation techniques of mRNA to extend the lifespan of RPTEC. ASO binds a specific sequence on target mRNA and blocks the proceeding of

**Table 1. Procedures of large-scale culture of lifespan-extended RPTEC**

1. Kidney cortex is collected from non-lesion parts and incubated with collagenase.
2. Dispersed renal tubular epithelial cells are collected, and proximal epithelial cells are isolated with anti-CD13 antibody conjugated with magnetic beads.
3. Purified RPTEC is seeded in a 225-cm² flask. Next day, living cells are subcultured in a new 225-cm² flask.
4. When subconfluent, cells are transfected with p16INK4a siRNA, and next day, subcultured into two 632-cm² chambers.
5. When subconfluent, cells are transfected with p16INK4a siRNA, and next day, subcultured into a 6320-cm² multilayer chamber and two 632-cm² chambers.
6. When subconfluent, cells in a 6320-cm² multilayer chamber are harvested and used for construction of bioartificial renal tubule devices. Cells in two 632-cm² chambers are transfected with p16INK4a siRNA, and next day, subcultured into a 6320-cm² multilayer chamber and two 632-cm² chambers.
7. Repeat Step 6 until cells become senescent or experiments over.

**Table 2. Process of a representative large culture**

<table>
<thead>
<tr>
<th>Culture period (Days)</th>
<th>Passage</th>
<th>Culture area (cm²)</th>
<th>Cell number (×10⁶)</th>
<th>Yields (×10⁶)</th>
<th>PD</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>225</td>
<td>4.1</td>
<td>3.9</td>
<td></td>
<td>CD13(+) cells from cortex</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>225</td>
<td>3.9</td>
<td>18.0</td>
<td>57.6</td>
<td>Transfected on the previous day</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>632 × 2</td>
<td>18.0</td>
<td>48.0</td>
<td>9.6</td>
<td>Transfected on the previous day</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>6320</td>
<td>550.0</td>
<td>54.5</td>
<td>3.4</td>
<td>For bioartificial device construction</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>6320</td>
<td>45.4</td>
<td>9.1</td>
<td>328.0</td>
<td>Transfected on the previous day</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>632 × 2</td>
<td>46.5</td>
<td>9.3</td>
<td>55.8</td>
<td>Transfected on the previous day</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>6320</td>
<td>379.5</td>
<td>63.9</td>
<td>3.0</td>
<td>For bioartificial device construction</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>6320</td>
<td>53.3</td>
<td>10.7</td>
<td>501.5</td>
<td>Transfected on the previous day</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>632 × 2</td>
<td>41.3</td>
<td>8.3</td>
<td>49.5</td>
<td>For bioartificial device construction</td>
</tr>
<tr>
<td>9</td>
<td>7</td>
<td>632 × 2</td>
<td>648.0</td>
<td>72.5</td>
<td>3.9</td>
<td>For bioartificial device construction</td>
</tr>
<tr>
<td>10</td>
<td>8</td>
<td>6320</td>
<td>406.0</td>
<td>59.3</td>
<td>2.7</td>
<td>Transfected on the previous day</td>
</tr>
<tr>
<td>11</td>
<td>9</td>
<td>6320</td>
<td>330.0</td>
<td>67.5</td>
<td>2.7</td>
<td>Transfected on the previous day</td>
</tr>
<tr>
<td>12</td>
<td>10</td>
<td>632 × 2</td>
<td>298.5</td>
<td>49.5</td>
<td>2.4</td>
<td>Transfected on the previous day</td>
</tr>
</tbody>
</table>

Total 3.49 × 10⁹ cells (27.8 PD) for bioartificial device construction

*PD calculated from total cell number of one 6320-cm² chamber and two 632-cm² chambers.*
Fig. 5. Leakage and reabsorption in bioartificial renal tubule devices with lifespan-extended human RPTEC. Bioartificial renal tubule devices (0.8 m²) were prepared by seeding lifespan-extended human RPTEC on inner surface of hollow fibers as described in Subjects and Methods. For 17 days of circulation of culture medium, leakage and reabsorption were evaluated by single-pass perfusion for 30 min every other day. The mean ± SD of four different experiments was represented. (A) Leakages of creatinine and urea nitrogen. Medium containing creatinine and urea nitrogen was passed through the inner compartment of the device, and leakages to the outer compartment were measured. During the first week of incubation, leakages of urea nitrogen and creatinine through cell-defected area of hollow fibers decreased according to cell proliferation and stabilized at ~15% after Day 7. (B, C and D) Reabsorptions of water (B), glucose (C) and sodium (D). Movement of these molecules from the inner compartment to the outer compartment were measured. According to the decrease of leakage, reabsorptions increased during the first week of incubation. They reached the maximum levels and retained them after Day 7.

Fig. 6. Scanning electron micrographs of lifespan-extended RPTEC on hollow fibers. Hollow fibers were retrieved from bioartificial renal tubule devices after examination of leakage and reabsorption. (A) Low magnification (×300); scale bar = 100 μm. (B) High magnification (×1000); scale bar = 10 μm.

Fig. 7. Metabolisms of β2-microglobulin and pentosidine in bioartificial renal tubule mini-devices. Bioartificial renal tubule mini-devices (65 cm²) were constructed with lifespan-extended RPTEC, and circulation of medium containing uremic toxins was performed, and samples were collected and measured as described in Subjects and Methods. At each time point, the mean ± SD of four different experiments was represented. (A) β2-microglobulin. (B) Pentosidine. Differences from backgrounds at each point were corresponded to amounts of metabolized pentosidine.
protein translation at that point [34–36]. In the case of RNAi, transfected synthetic double-stranded siRNA causes sequence-specific cutting and the following degradation of target mRNA [28, 29]. RNAi experiments showed more significant effects of lifespan extension. Treatments with siRNAs for p53 and p16INK4a showed over 30 PD and 60 significant effects of lifespan extension. Treatments with sequence-specific cutting and the following degradation of transfected synthetic double-stranded siRNA causes se-

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As to functional characteristics of lifespan-extended RPTEC, we found that expressions of characteristic genes of RPTEC (i.e. GGT1 and GULT1) recovered by culturing cells on porous membranes even after gene expression was decreased during replications on conventional plates. As suggested in our previous study [6], the expressions of these genes were enhanced by making osmotic pressure across membrane by 4% albumin.

As one immediate application of our approach, we con-

structed bioartificial renal tubule devices with lifespan-

extended RPTEC. Construction of one device requires a few 10^9 RPTEC [4, 37]. RPTEC should be controllable in growth [38], and functionally active and safe for clinical use. These requirements are satisfied by the lifespan-extended human RPTEC supplied by p16INK4a siRNA transfection. For construction of one device, we seeded 3–7 x 10^8 cells and circulated culture media to proliferate cells until they covered the entire inner surface of hollow fibers. The confluence of cells was monitored by leakages of creatinine and urea nitrogen added into the inner compartment of the device, and reabsorptions of water, sodium and glucose were examined at the same time. During the first week of incubation, leakages decreased gradually from ~30% and settled at ~15% after Day 7. In our previous report in which LLC-PK1, a line cell of porcine RPTEC, was seeded on polysulfone hollow fibers, leakages similarly decreased and stabilized at ~10% [6]. Since the confluence of cells in hollow fibers was confirmed by SEM as represented in Figure 6, these fundamental leakages were thought to be caused by ‘leaky epithelia’ made of RPTEC. Urea nitrogen and creatinine were thought to move through intercellular spaces with water even after the confluence of cells was established. Coincident with the completion of confluent monolayer of cells in hollow fibers, reabsorptions also stabilized at maximum levels. During the stabilized period, reabsorptions of water and sodium were compara-

t ble to those of our previous report, while reabsorption of glucose was about half of that shown by LLC-PK1 devices. Additionally, experiments with bioartificial renal tubule mini-devices represented active metabolism of beta-microglobulin and pentosidine by lifespan-extended RPTEC. These data indicated good performances of lifespan-extended human RPTEC seeded in hollow fibers made of EVAL membrane. All these results indicated the high per-

formance and the significant viability of lifespan-extended RPTEC seeded in hollow fibers in the bioartificial renal tubule device.

Previously, we estimated the total amounts of water, so-

dium and glucose transported across LLC-PK1 cell layer-

attached hollow fiber devices with 0.8 m^2 of the surface area for 24 h [39]. The transported amounts of water and sodium were ~90% and 93% of the targeted amounts per day, respectively, which was almost the same as those in 6 L of ultrafiltrate from uremic patients. The amount of transported glucose, on the other hand, was 214% of the target amount per day. These and the present results suggest that filtrate from dialysis patients could be regenerated partially by the bioartificial tubule device, although complete substitution with regenerated filtrate may be difficult in each hemofiltration treatment. In the treatments of patients with AKI and of chronic hemodialysis patients, the bioartificial tubule device is considered to play important roles to prevent the progress of inflammatory condition by improving oxidative stress and other unexpected roles of RPTEC [40].

The recent progress of studies of embryonic stem cells and inducible pluripotent stem cells are expected to con-
 tribute to the development of regenerative medicine. Cell transplantation therapy with these stem cells may solve the problem of the serious shortage of donor organs for the patients with end-stage renal failure in the future [41]. However, there are many technical and ethical hurdles to overcome before regenerative medicine with kidney stem cells can be established. Contrarily, therapy with the bio-

artificial renal tubule device has the possibility to become a usable means to fill the immediate need for both AKI and CKD, if a large number of highly functional RPTEC could be constantly provided. In this study, we presented safe and effective methods to supply a large number of RPTEC by expanding the replicative lifespan of cells in culture. Read-

ily applicable whenever RPTEC are isolated, our approach will contribute also to basic research and tissue engineering in the field of nephrology.

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