Development of a New Conditionally Immortalized Human Liver Sinusoidal Endothelial Cells

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Liver sinusoidal endothelial cells (LSECs), which are specialized endothelial cells that line liver sinusoids, have been reported to participate in a variety of liver functions, such as blood macromolecule clearance and factor VIII production. In addition, LSECs play crucial roles in liver regeneration following acute liver injury, as well as the development and progression of liver diseases or drug-induced hepatotoxicity. However, the molecular mechanisms underlying their roles remain mostly unknown. Therefore, in order to contribute to the clarification of those mechanisms, herein we report on the development of a new immortalized human LSEC (HLSEC) line. To produce this cell line, two immortalized genes were introduced into the primary HLSECs, which eventually resulted in the establishment of the HLSEC/conditionally immortalized, clone-J (HLSEC/ciJ). Consistent with the two-immortalized gene expression, HLSEC/ciJ showed excellent proliferation activity. Additionally, the results of gene expression analyses showed that several LSEC (as well as pan-endothelial) marker mRNAs and proteins were clearly expressed in HLSEC/ciJ. Furthermore, we found that adherence junction proteins were localized at the cell border in the HLSEC/ciJ monolayer, and that the cells exhibited a tube-like structure formation property. Taken together, the results obtained thus far indicate that we have successfully immortalized HLSECs, resulting in creation of HLSEC/ciJ, a cell line that possesses infinite proliferation ability while retaining possession of at least some HLSEC features. We believe that the HLSEC/ciJ have the potential to provide a valuable and unlimited alternative source of HLSECs for use in liver/LSEC physiology/pathophysiology, pharmacology, and toxicology studies.

Key words liver sinusoidal endothelial cell; immortalized cell; in vitro liver model; liver

LSECs have drawn significant attention in various liver study fields, where they are expected to provide novel insights into liver physiology/pathophysiology, as well as crucial targets for drug development or hepatotoxicity.

It has been considered likely that LSEC fulfill their unique roles through reciprocal cross-talk with other hepatic cells, specifically hepatocytes, Kupffer cells, and hepatic stellate cells. Having said that, it is also true that molecular entities and pathways responsible for LSEC functions, along with mechanisms underlying communications between LSEC and other hepatic cells, remain mostly unknown. To facilitate clarification of these points, especially in humans, it is likely that in vitro human LSEC models will provide essential experimental tools. As such, primary human LSEC (pHLSEC), as well as their co-cultured model with hepatocytes, have been used, for example, in studies for viral infection, liver immunity, and LSEC physiology. However, the fact that pHLSEC are prone to degeneration, difficult to obtain, and time-consuming to prepare, significantly hinders their use in various LSEC studies.

Therefore, in order to develop a useful in vitro LSEC model, we have focused on cell immortalization, one way to accomplish which is the introduction of the two genes, temperature-sensitive simian virus 40 large T antigen (tsSV40T) and the human telomerase reverse transcriptase catalytic subunit (hTERT), into LSECs. tsSV40T can drive cell prolifera-
tion in the permissive temperature (33°C), while it undergoes destabilization to disappear at non-permissive temperatures (above 37°C). hTERT prevents telomere DNA shortening, which allow the cells to proliferate infinitely.

Bearing in mind the background described above, herein we report on the development of a new immortalized HLSEC line that is expected to provide a useful tool for various HLSEC studies.

MATERIALS AND METHODS

**Immortalization of Human Primary Liver Sinusoidal Endothelial Cells** Cryopreserved prHLSECs were obtained from DS Pharma Biomedical (Osaka, Japan). The cells were cultured at 37°C with 5% CO₂/95% air in CSC-Complete Defined Medium (Cell Systems Corporation, Kirkland, WA, U.S.A.) with 2% (v/v) Defined Cell Culture Boost (Cell Systems Corporation) and penicillin–streptomycin. Cell culture dishes were coated with type-I collagen (Sigma, St. Louis, MO, U.S.A.).

Immortalization of prHLSEC was performed using the temperature-sensitive simian virus 40 large tumor-antigen (tsSV40T) gene and the human telomerase catalytic subunit (hTERT) gene by the lentiviral technique as previously described. The immortalized HLSEC was grown on type-I collagen-coated dishes in the Vasculife VEGF Endothelial Medium (Discovery Labware, Woburn, MA, U.S.A.) containing 4 μg/mL basic fibroblast S at 33°C with 5% CO₂/95% air.

**Cell Proliferation Analysis** The proliferation ability of immortalized HLSEC was examined by direct cell counting. The cells were seeded at 1×10⁵ cells/mL (day 0) on a type-I collagen-coated 24-well culture plate, and the numbers of cells at three, five and nine days were determined using a cytometer.

**Total RNA Extraction, cDNA Synthesis, RT-PCR** Total RNA from immortalized HLSEC and prHLSEC were extracted using the ISOGEN with Spin Column (Nippon Gene, Tokyo, Japan) according to the manufacturer’s protocol. cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.) with random hexamer primers.

RT-PCR was performed using the primers shown in supplementary Table S1. The mRNAs of interest are listed in the figure legends.

**Immunocytochemistry** Immunocytochemistry was performed essentially based on the method described previously. The primary and secondary antibodies used are summarized in supplementary Table S2.

**Tube Formation Assay** The tube formation ability of immortalized HLSEC was assessed using a Matrigel-based culture method. The cells (5.0×10⁵ cells) were seeded on Matrigel (Discovery Labware, Woburn, MA, U.S.A.) coated 24-well culture plate and cultured at 33°C for 24h, after which the tube formation was observed under the microscope.

**Others** Method details and other information are provided in the supplementary materials.

RESULTS AND DISCUSSION

As illustrated in Fig. 1A, we first introduced the two immortalized genes into prHLSEC, and then isolated approximately 100 clones. From among these, based on screening results of a series of examinations (i.e., analyses for morphology, growth rate, survival rate, and mRNA expression profile), we isolated the clone-J that showed the most promising LSEC characteristics, such as morphological similarity with prHLSEC (Fig. 1B). (See also the supplementary materials including supplementary Fig. S1). Therefore, our subsequent characterization efforts focused solely on this clone, which is hereafter referred to as HLSEC/conditionally immortalized, clone-J (HLSEC/ciJ).

With the population doubling time of 75.8h, HLSEC/ciJ clearly showed extensive proliferation capabilities (Fig. 1C), and that proliferation rate was maintained over 50 successive passages. This was in clear contrast to the lack of significant proliferation shown by prHLSEC. Along with its aggressive proliferation property, mRNA and protein expression for tsSV40T and hTERT were clearly detected in HLSEC/ciJ by RT-PCR and immunocytochemistry, respectively (Fig. 1D).

Since they are intended for use as an in vitro HSEC model, HLSEC/ciJ are expected to retain HLSEC properties. Because HLSEC are specialized endothelial cells, they carry distinctive gene expression signatures, in addition to pan-endothelial marker gene expressions. Therefore, we examined both LSEC and endothelial marker mRNA expression in HLSEC/ciJ via RT-PCR. The results showed that mRNA expression of representative LSEC markers (such as lympathic vessel endothelial hyaluronan receptor 1 [LYVE-1], stabilin-1 and -2, junctional adhesion molecule-C [JAM-C], and Factor VIII), along with those of pan-endothelial markers (such as vascular endothelial-cadherin [VE-cadherin] and von Willebrand factor [vWF]), were clearly present in the cells (Fig. 2A). Furthermore, protein expression of some of those markers were also observed in the HLSEC/ciJ monolayer (Fig. 2B).

**Others** Method details and other information are provided in the supplementary materials.
lished. However, there is another HLSEC-derived immortalized cell line, known as TMNK-1, which has been immortalized by wild-type SV40T and hTERT. In addition to the fact that they are derived from the same cell-type, both HSEC/ciJ and TMNK-1 carry reversible immortalization systems (the former is mediated by the temperature-sensitive nature of tsSV40T, and the latter by the Cre/loxP system). Nevertheless, although no direct comparison has yet been made, there are supposedly several distinctive features with each cell line. This is because of differences in the genetic background of cell origins, clone isolation criteria, and culture conditions between the two cell lines. In actuality, our experiences have shown that each HSEC/ciJ clone has a unique gene expression profile (partially presented in Fig. S1). Therefore, the two HLSEC immortalized cell lines can be expected to play compensatory or mutually reinforcing roles in HLSEC studies as researchers take advantage of each line’s distinctive feature.

Since the HSEC/ciJ line has just been established, and since the present findings are apparently insufficient to delineate overall picture of their functional properties, additional and comprehensive research efforts aimed at their in-depth characterization will be necessary to identify the ways whereby HSEC/ciJ can be expected to contribute significantly to expanding knowledge related to LSEC and/or liver physiology/pathophysiology, toxicology, and pharmacology.

However, regarding how HSEC/ciJ can be utilized, it is possible that our cells will be valuable in the development of in vitro liver models co-cultured with hepatocytes, as well as other hepatic cells. This is based on the results obtained from our preliminary co-culture experiments with rat primary hepatocytes using a trans-well system, which show that, in addition to albumin mRNA, expression levels of several CYP mRNA (representative drug metabolizing enzymes) and organic anion transporting polypeptide mRNAs (representative hepatic drug uptake transporters) display a trend of increase in co-cultured hepatocytes (supplementary Fig. S2). Therefore, HSEC/ciJ has the potential to become a useful component of in vitro liver model for hepatic drug metabolism/uptake and toxicological studies. Furthermore, while noting that the trans-well system used in this study does not allow the cells to come into direct contact with each other due to the space between the insert and the well, the results obtained point to the possibility that HSEC/ciJ are at least capable of enhancing hepatocyte functions through releasing (or exchanging)
trophic factors. Therefore, HLSEC/ciJ can be expected to contribute to clarification of the molecular mechanisms behind hepatocyte-LSEC communications within the liver.

In order to further increase their usefulness in various HLSEC studies, it will be important to find ways to improve HLSEC/ciJ functionality. One such possibility may be the elimination of immortalization signal by shifting culture temperature to a non-permissive level (over 37°C), which is expected to result in the stimulation of cell differentiation. Another potential method might be the modification of culture conditions (such as depletion of serum and/or other growth factors). Since we have found that either or both approaches have been effective for enhancing their cellular differentiation properties in other temperature-sensitive immortalized human cells (HBMEC/ciβ, HASTR/ci35 and HBPC/ci37) (and unpublished observations), it will definitely be worthwhile to determine whether these approaches can also be applied to HLSEC/ciJ.

To summarize, we have developed a new line of conditionally immortalized HLSEC, which we have named HLSEC/ciJ, that shows infinite proliferation ability, while concomitantly exhibiting LSEC-specific mRNA expression profile, and which can be expected to provide an unlimited and useful tool for HLSEC studies. Since HLSEC have drawn significant attention in various fields of liver sciences, including liver regeneration, fibrosis, and drug-induced hepatotoxicity, it is hoped that HLSEC/ciJ will significantly contribute to facilitating such studies. To this end, further HLSEC/ciJ characterization will be warranted.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

Fig. 2. LSEC and Endothelial Marker Gene Expression Profiles of HLSEC/ciJ

A, The mRNA expression profile of HLSEC/ciJ were examined and compared with that of pHLSEC. The genes of interest are: LSEC markers (LYVE-1, stabilin-1, stabilin-2, Fc-gamma receptor IIb [FCGR2B, also known as CD32B or SE-1], FactorVIII, liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin [L-SIGN]; liver endothelial differentiation-associated protein-1 [Leda-1], JAM-C, endothelial markers (β-catenin, ZO-1, VE-cadherin, vWF, CD31, CD34, intercellular adhesion molecule-1 [ICAM-1]), endocytosis genes (EH domain containing 3 [Ehd3] and Caveolin-1 [CAV-1]), transcription factors (Tfec and LIM domain only 3 [Lmo3]), cytokine and growth factors (C-X-C chemokine receptor type 4 [CXCR4], vascular endothelial growth factor receptor 1 [VEGFR1], VEGFR3, and Wnt3), immune system genes (Toll-like receptor 4 [TLR4], and CD31), and the cytoskeletal organization gene (Rho family GTPase 3 [Rnd3]). GAPDH mRNA was used as an internal control. NTC refers to non-template control. B, The protein expression of LSEC and endothelial marker genes in HLSEC/ciJ were examined by immunocytochemistry. HLSEC/ciJ were cultured for 10d to develop a monolayer. C, OATP1B1, OATP1B3, and VEGF-A mRNA expression were examined in HLSEC/ciJ cells. OATP1B1 and OATP1B3 mRNA expression are known to be hepatocyte-specific, and VEGF-A mRNA expression has been reported to be very low in pHLSEC. Caco-2 and human primary hepatocytes cDNA were used as positive control. HPH indicates human primary hepatocytes. NTC refers to non-template control. D, the tube formation property of HLSEC/ciJ was examined. HLSEC/ciJ was seeded onto a Matrigel-coated dish to allow tube-like formation. In the above examinations, the experiments were performed three times, and the representative results are shown.
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