**Oxygen-permeable membrane-based direct oxygenation
remarkably enhances functions and gene expressions of rat
hepatocytes in both 3D and sandwich cultures**

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Abstract

Although there have been remarkable progresses in hepatocytes cultures in terms of mimicking microenvironments of *in vivo* liver, oxygen supply is still a critical issue. In this study, we investigated the effect of direct oxygenation through oxygen-permeable membranes on functionalities of hepatocytes in two widely accepted advanced culture models, sandwich culture and 3D culture. Rat hepatocytes were cultured on the polydimethylsiloxane (PDMS) membranes for 14 days in monolayer culture, sandwich culture with Matrigel and 3D culture with microporous expanded polytetrafluoroethylene (ePTFE) membranes in the presence and absence of direct oxygenation from the other side of the membranes. The present results showed remarkable enhancement of hepatocytes duration and their functions by oxygen transfer through PDMS membranes in all these three cultures. The hepatocytes cultured in sandwich with oxygen exhibited extended survival and highest maintenance of metabolic activities, such as albumin productivity and Cyp1a1/2 activity. Additionally, the expression levels of various drug-metabolism genes, as examined by PCR arrays, were also closest to those of freshly isolated hepatocytes. As the cellular maintenance has been greatly improved by microporous ePTFE membranes, the hepatocytes in 3D culture performed increased functions that comparable to those in sandwich culture. This study clearly illustrates that oxygenation is a critical factor to be considered in optimization of the microenvironments of hepatocytes cultures.

Keywords: tissue cell culture; oxygen transfer; microporous membrane; sandwich culture; 3D culture; optimization

1. Introduction

The liver acts as the center of metabolism and is indeed one of the most important sites of drug ADME (absorption, distribution, metabolism and excretion)-Efficiency/Toxicity. With the majority of liver functions, including xenobiotic biotransformation, performed by hepatocytes, hepatocyte-based *in vitro* models are currently being used in the drug development. Consequently, the development and characterization of long-term cultures of hepatocytes as *in vitro* experimental models represent an important goal. A major problem, however, related to the hepatocytes and their *in vitro* cultures is the progressive loss of the hepatocellular phenotype in conventional monolayer cultures, at both morphological and functional levels [1,2]. To overcome these effects, two widely accepted cellular culture models have been developed, sandwich culture model and 3D culture model (such as spheroid culture model), to restore the metabolic functions over extended periods of cultivation [3], with advanced substrate [4] and further stimulation by adding suitable exogenous factors [5].

Sandwich culture has been a valid cellular model for the hepatocytes relying on the restoration of cell-extracellular matrix (ECM) contacts, which is a one of the key roles in the culture configurations of primary hepatocytes [4,6]. Using an overlay of Matrigel, a basement membrane extract from Engelbreth-Holm-Swarm (EHS) mouse sarcoma, could enable cells to express high functions while simplifying the culture model compared to the standard sandwich with two gel layers of type I collagen [7,8]. More recently, another advanced format, in which hepatocytes could be cultured in a 3D configuration to represent the cell-to-cell contact, has been developed. In 3D culture (such as spheroid culture model), the hepatocytes maintain not only *in vivo*-like morphological characteristics, but also liver-specific functionalities [9,10]. However, decreased functionalities were previously observed compared to those in sandwich culture, due to deficient cellular maintenance [11]. Expanded

polytetrafluoroethylene (ePTFE), a microporous synthetic polymer, is one of the preferable substrates used to improve stability in culture according to firmly attached hemispheroids [12–14]. Based on ePTFE membranes, the quantitative comparison of hepatocyte functions between 3D culture and sandwich culture is highly valuable.

Oxygen is one of the critical exogenous factors for the hepatocytes *in vitro*. The oxygen consumption rate (OCR) of rat hepatocytes is quite high, almost 10-fold higher than that of other cell types, and 3-fold more during the attachment, spreading and reorganizing process right after the inoculation [15]. However, conventional tissue culture plates, such as the tissue culture treated polystyrene (TCPS), are incapable of sufficient oxygen supply as a result of the limited solubility of oxygen in the culture media, which force the hepatocytes to undergo hypoxia [16,17]. In our previous work, to solve this problem, we have developed a simple method of culturing cells on an oxygen-permeable membrane, polydimethylsiloxane (PDMS), to meet this intensive requirement for oxygen. Although there are several hepatocyte cultures using PDMS-based devices, such as microfluidic, PDMS bioreactors are integrated on the glass substrate. Thus, the diffusion through culture medium or not well-controlled perfusion is still likely to cause oxygen shortage at the cell layer. Our study of culturing hepatocytes direct on a PDMS substrate has barely been made [18]. The PDMS membranes could facilitate direct oxygenation by diffusion through the membranes, which is expected to meet the cellular OCR requirements and increase functional capabilities. As governed by Henry's law, the actual oxygen concentration at the cell level could be very close to the ambient concentrations [19,20].

In this study, we investigated the effect of direct oxygenation using PDMS membranes on functionalities of hepatocytes in sandwich culture with Matrigel and 3D culture with ePTFE membranes, where the cellular oxygen requirements could be completely meet. Various functional characteristics of the hepatocytes in each culture were

systematically evaluated and compared. We aimed to explore a more reliable hepatocytes culture model with the sufficient oxygen supply to enhance hepatic metabolism and gene expression in a long duration for *in vitro* drug-screening research.

2. Materials and Methods

2.1 Rat hepatocyte isolation and high-/low-oxygen cultures

Primary hepatocytes were isolated from 7-8-week old male Wistar rats (Sankyo Laboratory, Japan) weighing 200-300 g using a two-step collagenase perfusion technique, as described previously [21]. Only those cells with viabilities exceeding 85% after isolation were used. All animals were treated in compliance with the University of Tokyo guidelines for animal experiments, following the guidelines of the Japanese Ministry of Education. Hepatocytes were suspended in ice-cold seeding medium (Williams E with 10% FBS) and seeded at a density of 1.0×10^5 cells/cm². The hepatocytes were maintained at 37°C in a humidified incubator in a 95% air and 5%CO₂ atmosphere. On day 1, the seeding medium containing unattached cells was aspirated, and culture medium containing Williams' Medium E (Gibco, Japan), 1% NEAA (Gibco, Japan), non-essential amino acids (Gibco, Japan), 0.1 µM insulin (Takara, Japan), 1 µM dexamethasone (Wako, Japan), 10 ng/ml mouse epidermal growth factor (EGF; Takara, Japan), 0.5 mM ascorbic acid 2-phosphate (from magnesium salt n-hydrate; Wako, Japan), 0.1 µM CuSO₄, 0.01 µM H₂SeO₃ and 1 µM ZnSO₄ was added. The Williams E-based culture medium was changed every two days.

To enable direct oxygenation by diffusion through PDMS membranes (high-oxygen cultures, PDMS-O₂ (+)), hepatocytes were cultured on a 0.5-mm-thick PDMS membrane assembled on a polycarbonate 24-well plate as Monolayer-O₂(+) culture (Vessel, Japan) (Fig. 1A). PDMS discs were cut up and inserted into the wells of TCPS plates (Iwaki, Japan) as Monolayer-O₂ (–) culture to control the surfaces consistent as the low-oxygen cultures

(PDMS-O₂ (-)) (Fig. 1B). Prior to cell culture, the PDMS surfaces were treated with oxygen plasma for 10 seconds, using a Reactive Ion Etching (RIE) machine (RIE-10NR; Samco, Japan), and coupled with aminosilane (Shinetsu Silicone, Japan). The introduced amino groups were reacted with the cross linker N-(4-Maleimidobutyryloxy) succinimide (GMBS; Dojindo, Japan) and coated with ocean collagen. Conventional monolayer culture on collagen coated TCPS plates was employed as a control (TCPS-O₂ (-)) (Iwaki, Japan) (Fig. 1B).

2.2 Sandwich cultures with Matrigel and 3D cultures with ePTFE membranes

The seeding medium containing unattached cells in both Monolayer-O₂ (+) culture and Monolayer-O₂ (-) culture was aspirated on day 1, and culture medium supplemented with 150 µg/ml Matrigel was added as sandwich cultures (Sandwich-O₂ (+), Sandwich-O₂ (-)) (Fig. 1B) [15]. The Matrigel-supplemented culture medium was also changed every two days.

ePTFE is a porous form of a synthetic fluoropolymer with a microstructure characterized by nodes interconnected by fibrils. The fibrils are length of 10-50 µm, interval of 2-5 µm, thickness of 50-70 µm and porosity of 90% (Vessel, Japan), which could restrict hepatocytes spread. The hepatocytes could initially attach on the ePTFE membranes and elongate to form cell layers by cell-to-cell connection. The elongated cells then started to retract and to form multicellular aggregates. The ePTFE membranes were washed with ethanol, and immersed in a coating solution of 1% poly-amino-acid urethane copolymer (PAU) and 0.1% ocean collagen in dichloroacetic acid, washed with water to remove the excess coating solution, dried, and sterilized with ethylene oxide [22]. Then ePTFE membranes were fixed on the PDMS membranes as a support, assembled on a polycarbonate 24-well plate (3D-O₂ (+)) (Vessel, Japan) (Fig. 1B). PDMS+ePTFE discs were cut up and inserted into the wells of TCPS plates (Iwaki, Japan) to keep the surfaces consistent as the

low-oxygen cultures (3D-O₂ (-)). Fig. 1C shows the scanning electron microscopy (SEM; SM-300; F1 Topcon, Japan) picture of ePTFE.

2.3 Vertical cross-section analysis

On day 1 and day 14, cell-loaded PDMS+ePTFE substrates in 3D cultures were rinsed with PBS. After 1 hour of fixation with 4% paraformaldehyde (PFA; Nacalai Tesque, Japan), the cell sheets on the PDMS+ePTFE membranes were rinsed with PBS (5 minutes, 3 times). Histological samples were prepared by the Saipaso Research Center (Tokyo, Japan) as follows: fixed samples were dehydrated, embedded in paraffin, then 5 μ m thin vertical sections were cut off, deparaffinized, rehydrated, and stained with Hematoxylin and Eosin (HE). Images were taken with a transmitted light microscope (BX50; Olympus, Japan).

2.4 DNA quantification

Extracted DNA was quantified from the inoculated cells suspension and adherent cells on day 1 and day 14, which were scraped off and recovered in 1 ml PBS after rinsing twice with PBS to remove the unattached cells. The samples were sonicated for 20 seconds at 40 W in an ice bath. The DNA concentrations were measured using 4', 6-diamidino-2-phenylindole (DAPI; Dojindo, Japan) fluorometry [23]. Data represent the means $\pm$ SD (n = 6) from two independent experiments.

2.5 Oxygen tension measurements and OCR calculations

The oxygen tension (pO₂) values encountered by the cells were measured with a fluorescent oxygen probe (PreSens, Germany) on day 2. The oxygen probe is a microsensor with a tapered tip based on a 140 μ m silica fiber and is available with a diameter < 50 μ m. The silica fiber with its oxygen-sensitive tip is protected inside a stainless steel needle and can be extended for measurement. The probe tip was set to a position at the surface of cell layer. The pO₂ was monitored until a steady state was obtained and recorded as the percentage of the partial pressure of the dissolved oxygen, which was approximately 20%

under ordinary atmosphere conditions.

To estimate the cellular OCR of the hepatocytes in different culture conditions, a one-dimensional diffusion reaction equation following Fick's second law was used to model the system (Eq. (1)), with the parameters listed in Table 1:

$$F = D \frac{(C - C_{\text{cell}})K}{Z} = \sigma a \quad , \quad (1)$$

Where we assumed the oxygen diffusion and consumption of the hepatocytes were at a steady state. F is the supplied oxygen flux (pmol/s/cm²); D is the diffusion coefficient of oxygen (cm²/s); C is the ambient oxygen partial pressure at the gas interface (mmHg); C_{cell} is the oxygen partial pressure at the cell layer (mmHg); K is the oxygen solubility in the media (pmol/ml/mmHg); Z is the depth of the diffusion path (cm); σ is the surface cell density (cells/cm²); a is the OCR (pmol/s/10⁶ cells). **Data represent the means $\pm$ SD (n = 3).**

2.6 Albumin production measurements

The amount of secreted albumin in the culture medium was measured by a sandwich-type enzyme-linked immunosorbent assay (ELISA) [24]. A goat anti-rat albumin (Cappel, CA) was used as the primary antibody, and a horseradish peroxidase-conjugated sheep anti-rat albumin (Cappel, CA) was used as the secondary antibody. **Data represent the means $\pm$ SD (n = 6) from two independent experiments.**

2.7 PCR array analysis

Freshly isolated hepatocytes **were used to obtain *in vivo* mRNA expression levels**, and the cultured hepatocytes on day 2 were used to obtain ***in vitro* mRNA expression levels**. Total RNA from hepatocytes was isolated using an RNeasy kit (QIAGEN, USA) **and DNA digestion (QIAGEN DNase Kit, USA)** and quantified by **measuring UV absorbance at 260 nm**. **1 μ g of total RNA was reverse transcribed in a final volume of 20 μ l to obtain cDNA via an RT² First Strand kit (SA Biosciences, USA), and the cDNA was diluted to 111 μ l by adding RNase-free water and stored at -20°C .** The mixture of appropriately diluted cDNA,

master mix and deionized water was pipetted into an RT² profiler PCR array and cycled (10 min at 95 °C, 15 s at 95 °C, 1 min at 60 °C for 40 cycles) in a StepOnePlus Real-Time PCR System (ABI, USA). The PCR array was used to simultaneously examine the mRNA levels of 89 genes, including five housekeeping genes, known to be involved in rat drug-metabolism, using a 96-well plate according to the manufacturer's protocol (PARN-002Z; QIAGEN, USA).

2.8 Cytochrome P450 assay

The 7-ethoxyresorufin-O-deethylase assay (EROD) is a measurement of the deethylation activity of cytochrome P450 1A-associated mono-oxygenase enzymes. The assay was initiated by incubating the cultures with 10 µM 7-ethoxyresorufin and 10 µM dicumarol in culture medium at 37°C for 1 hour. The amount of resorufin converted by the enzymes was calculated by measuring the fluorescence in the incubation medium at 530 nm (excitation)/585 nm (emission) using a microplate reader (FP3000, Microtec Niton) and comparing the samples to standards processed using the same method. Data represent the means ± SD (n = 6) from two independent experiments.

2.9 Statistical analyses

The data are represented as the mean ± SD. An analysis using ANOVA and Tukey's test was performed to determine the statistical significance of the results, with values of $P < 0.01$ and $P < 0.05$ considered to be highly significant and significant, respectively.

3. Results

3.1 Changes in rat hepatocyte morphology and viability

On day 1, the hepatocytes cultured in TCPS-O₂ (-), Monolayer-O₂ (-) and Sandwich-O₂ (-) initially formed discontinuous monolayers (Fig. 2A). A rapid detachment of cells was next observed and the hepatocytes were obviously dead on day 14, especially in the TCPS-O₂ (-) culture (Fig. 2B). In 3D-O₂ (-), the spreading of hepatocytes was limited by ePTFE

fibrils. The cells retained their cuboidal shape and self-assemble themselves into spheroids within 1 day (Fig. 2A). The size of spheroids seemed to increase and the cells largely detached on day 14 (Fig. 2B). In contrast, the hepatocytes cultured in Monolayer-O₂ (+) and Sandwich-O₂ (+) spread into a completely confluent monolayer on day 1 (Fig. 2A). Comparing with those in Monolayer-O₂ (+), the cells in Sandwich-O₂ (+) were more tightly adhered to the PDMS membranes on day 14 (Fig. 2B). In 3D-O₂ (+) culture, the highly multilayered cell aggregates appeared on day 1 (Fig. 2A), and gradually formed into hemispheroids. The cells still maintained large attachment on the surface of ePTFE membranes on day 14 (Fig. 2B).

3.2 Histological analysis of vertical cross-sections

Hematoxylin-eosin stained sections clearly show the morphology of hepatocytes in 3D cultures. In both 3D-O₂ (-) and 3D-O₂ (+), the hepatocytes formed both multilayers and spheroids throughout the culture period. Comparing to the monolayer in 3D-O₂ (-), the cells cultured in 3D-O₂ (+) initially formed double layers from day 1 and organized into spheroids in a larger size, where the cells exhibited a cubical shape (Fig. 3A). On day 14, much less denuded cells inner the spheroids were observed in 3D-O₂ (+) than those in 3D-O₂ (-), and cells formed hemispheroids (Fig. 3B).

3.3 Cellular maintenance in terms of DNA amounts

On Day 1, there is no significant difference of the DNA amounts of attached cells in all the cultures (Fig. 4). On Day 14, the DNA amounts remained remarkably higher in the PDMS-O₂ (+) cultures compared with the residual cells in the TCPS-O₂ (-) culture and the PDMS-O₂ (-) cultures. The DNA amounts exhibited nearly the same tendency in both the PDMS-O₂ (-) cultures and the PDMS-O₂ (+) cultures, where the hepatocytes cultured in Sandwich exhibited the highest attach capability, followed by 3D cultures as the second.

3.4 Oxygen tension at the cell level and OCR values

Three runs of pO_2 changes within 60 minutes are shown in Fig. 5A-C. On day 2, the pO_2 level was around 9.3%, 10.2% and 10.8% in the TCPS- O_2 (-) culture; 9.1%, 11.4% and 10.4% in Monolayer- O_2 (-); 10.1%, 10.3% and 9.7% in Sandwich- O_2 (-); and 10.3%, 10.3% and 9.9% in 3D- O_2 (-), respectively. In contrast, the pO_2 level stabilized at 19.3%, 19.1% and 19.6% in Monolayer- O_2 (+); 18.9%, 19.6% and 19.4% in Sandwich- O_2 (+); and 19.3%, 19.0% and 19.5% in 3D- O_2 (+), respectively. These data demonstrated that the dissolved oxygen tension accurately represented the ambient partial pressure levels through PDMS or PDMS+ePTFE membranes.

The OCR values for the hepatocytes were shown in Fig. 6. For the cells cultured in the TCPS- O_2 (-) and the PDMS- O_2 (-), the oxygen diffusion through polystyrene was neglected, as this polymer is considered to be oxygen impermeable. The cells were supplied with oxygen that dissolved at the air-liquid interface and was transported to the cells through culture medium. Based on the Eq. (1) and Table 1, the OCR values obtaining from pO_2 were determined to equal 71.6 pmol/s/ 10^6 cells in the TCPS- O_2 (-) culture, and 70.2, 72.1 and 71.1 pmol/s/ 10^6 cells in Monolayer- O_2 (-), Sandwich- O_2 (-) and 3D- O_2 (-), respectively, on day 2. Considering a ~ 91.3 -fold increase in the oxygen transfer flux from the culture medium (14.5 pmol/s/cm²) to the PDMS membranes (1321.2 pmol/s/cm²) (Eq. (1), assuming that the oxygen concentration becomes 0 pmol/ml at the cell layer), the cellular oxygen consumption resulted primarily from the flux through the bottom in the O_2 (+) cultures [20]. The OCR values significantly increased to 440.4, 462.4 and 484.4 pmol/s/ 10^6 cells in Monolayer- O_2 (+), Sandwich- O_2 (+) and 3D- O_2 (+) conditions, respectively. These large variations in respiratory activity among the TCPS- O_2 (-) culture, the PDMS- O_2 (-) cultures and the PDMS- O_2 (+) cultures have commonly been attributed to ‘hepatic hibernation’, a feedback mechanism that allows hepatocytes to maintain their viability by decreasing oxygen consumption and respiration circuits in hypoxic environments [25,26].

3.5 Albumin production

Comparing with the cells cultured on the PDMS membranes, the albumin production remained far lower throughout the cultivation period in the TCPS-O₂ (-) culture and the PDMS-O₂ (-) cultures. In Sandwich-O₂ (-), the albumin production reached a maximum value on day 5 and then gradually decreased (Fig. 7). There is no obvious difference of the albumin production between 3D-O₂ (-) and Monolayer-O₂ (-). This low performance particularly happened on the ePTFE membrane in the PMDS-O₂ (-) condition, due to the poor cellular maintenance. The spheroids formed on day 1 rapidly detached or re-organized into spheroids with a much bigger size, in which the hepatocytes, especially the inner cells, probably died. Cultures in the PDMS-O₂ (+) sustained high albumin production from day 1. The hepatocytes cultured in Sandwich-O₂ (+) exhibited the highest albumin secretion capability, peaking on day 11, and maintaining up to 28.5-fold higher values than those of cells cultured in the TCPS-O₂ (-) on day 14. The cells cultured in 3D-O₂ (+) also exhibited increased performance that comparable to those in Sandwich-O₂ (+), peaking on day 5, and maintaining up to 20.8-fold higher values than those of cells cultured in TCPS-O₂ (-) on day 14.

3.6 mRNA expression of enzymes involved in drug-metabolism

The mRNA expression profiles of 84 genes involved in drug-metabolism, including drug transporters (6 genes), phase I (14 genes) and phase II metabolism enzymes (56 genes) and several other genes (8 genes), were determined in differently cultured hepatocytes using PCR arrays. The average mRNA expression levels in the TCPS-O₂ (-) culture were normalized to 1 for all measured genes (Table 2). Gene expression levels that were 2-fold greater than these normalized values included 42 genes, 55 genes and 54 genes in Monolayer-O₂ (-), Sandwich-O₂ (-) and 3D-O₂ (-), respectively, and increased to 59 genes, 63 genes, and 62 genes in Monolayer-O₂ (+), Sandwich-O₂ (+) and 3D-O₂ (+), respectively, when direct

oxygenation was applied. The expression of these genes was most obviously up-regulated. For the drug transporters, the genes expression of all the ATP-binding cassette family was largely increased in the PDMS-O₂ (+) cultures, including Abcb1b, Abcb1a, Abcb4 and Abcc1. For the Cyp family (phase I metabolizing enzymes), the genes expression was generally increased, especially in the PDMS-O₂ (+) cultures (11 genes out of 14, except Cyp17a1, Cyp1b1, Cyp2c7). For the phase II metabolizing enzymes, the genes expression of 2 carboxylesterases enzymes (Ces1e, Ces2c), 2 dehydrogenases enzymes (Hsd17b1, Hsd17b2), 2 glutathione peroxidases enzymes (Gpx3, Gpx5), 3 hydrolases enzymes (Ephx1, Faah, Fbp1), 1 kinases enzymes (Hk2), 4 oxidoreductases enzymes (Gsr, Nos2, Nos3, Nqo1), 1 paraoxonases enzymes (Pon3), 12 glutathione s-transferases enzymes (Chst1, Gsta1, Gsta4, Gstm1, Gstm2, Gstm3, Gstm4, Gstm5, Gstt1, Mgst1, Mgst2, Mgst3) and 1 transferases enzymes (Nat1) was increased in the PDMS-O₂ (+) cultures. These results indicated that the sufficient oxygen supply through PDMS membranes enhanced the expression of genes encoding various enzymes involved in drug-metabolism. Besides, with the application of Sandwich-O₂ (+) and 3D-O₂ (+), the majority of genes showed higher expression than those in Monolayer-O₂ (+).

The gene expression levels involved in drug-metabolism of cultured hepatocytes were next compared with those of freshly isolated hepatocytes using the same array (Table 3). The cells cultured in the PDMS-O₂ (+) exhibited remarkably higher gene expression levels than those grown in the TCPS-O₂ (-) culture and the PDMS-O₂ (-) cultures, where the genes expression was largely decreased. Only minor differences were observed between Sandwich-O₂ (+) and 3D-O₂ (+), in which 48 genes and 47 genes, respectively, maintained highest levels of transcription that were similar to those observed in rat liver. Comparing with freshly isolated hepatocytes, gene expression levels that had more than a 4-fold change were mostly down-regulated in the cultured cells (Supplement, Table 1), indicating that the *in vivo*-like

metabolism functions were still suppressed in these advanced *in vitro* cultures with direct oxygenation done in this study.

3.7 Cyp1a1/2 activity

With the Cyp family genes encoding phase I metabolizing enzymes showing increased expression in the PDMS-O₂ (+) cultures, we subsequently examined Cyp1a1/2 activity levels and normalized to the DNA amounts on day 1 (Fig. 8). In the TCPS-O₂ (-) culture and the PDMS-O₂ (-) cultures, the expression of Cyp1a1/2 was quite low, especially in the TCPS-O₂ (-) culture, decreased over the entire cultivation period. In contrast, the cells cultured in the PDMS-O₂ (+) exhibited enhanced Cyp1a1/2 activity, consistent with the increased levels of mRNA encoding these compounds (Table 2). From day 2 and then throughout the course of the experiment, the cells cultured in Sandwich-O₂ (+) exhibited the highest Cyp1a1/2 activity, peaking on day 8 and maintaining a value that was 23.8-fold significantly higher than the value observed in the TCPS-O₂ (-) culture on day 14. The cells cultured in 3D-O₂ (+) also exhibited the increased performance comparable to those in Sandwich-O₂ (+), peaking on day 8, and maintaining up to 18.1-fold higher values than those of cells cultured in TCPS-O₂ (-) on day 14.

4. Discussion

In this study, the effects of using PDMS membranes in two advanced culture models, sandwich culture and 3D culture, were examined to determine whether improved oxygen supply that completely meet the cellular OCR could induce significant metabolic and functional changes in rat hepatocytes. By accessing to the surrounding atmosphere, the PDMS membranes could regulate the actual oxygen concentrations at the cellular level. On the basis of the direct oxygenation through PDMS membranes, the hepatocytes cultured in both Sandwich with Matrigel and 3D with ePTFE membranes exhibited improved survival,

maintained remarkable expression levels of genes involved in drug-metabolism, as well high hepatic functionalities. A direct comparison of these two culture models affecting hepatocytes functions in a long duration for *in vitro* drug-screening research was therefore presented in this study.

The oxygen transport through PDMS membranes could completely meet the cellular OCR requirements in both sandwich culture and 3D culture, as well monolayer culture, which contributed to the great enhancement of various hepatic functions. Oxygen supply of the hepatocytes is a critical issue that is dramatically different between *in vivo* and *in vitro* [17,27]. Some strategies discussed in the literature involves the introduction of a high oxygen tension, such as a 95% O₂/5% CO₂ gas mixture [27]. Yet, atmospheres with such high oxygen partial pressures may easily promote the formation of reactive oxygen species [28]. Besides, the actual oxygen concentration encountered by cells or the cellular OCR was not assessed. In contrast, as shown by the direct measurement of oxygen tension at the cellular level, we demonstrated that PDMS-O₂(+) accurately represented the ambient partial pressure levels (Fig. 5), to maintain the OCR values ranging from 440.4 pmol/s/10⁶ cells (Monolayer-O₂(+) culture) to 484.4 pmol/s/10⁶ cells (3D-O₂(+) culture) (Fig. 6), values that are comparable to the *in vivo* liver cellular OCR (477 pmol/s/10⁶ cells) [17]. In the TCPS-O₂(-) culture and the PDMS-O₂(-) cultures, the low solubility of oxygen in the culture medium offers more than 10-fold lower oxygen to the cells compared with *in vivo* level [29,30], which resulted in 6-7-fold lowered OCR values (Fig. 6), indicating that the hepatocytes were experiencing extremely anaerobic conditions. Such hypoxic influence restricted hepatocytes to subconfluent cultures and suppressed metabolic activities.

3D culture in our study significantly prolonged the duration of viability and increased the functionalities of rat hepatocytes owing to ePTFE membranes, comparable to sandwich culture using matrigel. In 3D culture, as aspired by mimicking the structural and functional

characteristics of the hepatocytes *in vivo*, cohesive interactions between cells are greater than the adhesive interactions between the cells and culture substrate [31,32], which help spheroids to attain closely packed morphology. Non-adherent culture surfaces can be made by coating with agarose thin films [33] or hydrophobic polymers like poly (2-hydroxyethyl methacrylate) (PHEMA) [34] for generating Spheroids. Whereas, the spheroids formed on such non-adherent surfaces are more prone to be dislodged, accompanied with shortened cultivation period and lowered metabolic activities [31]. Besides, another problem is a broad size distribution of spheroids generated. The present results showed that ePTFE membranes improved the stability of 3D culture in terms of cellular maintenance with firmly attached hemi-spheroids in two weeks duration (Fig. 2-3), and dramatically enhanced liver-specific functions compared with monolayer culture (Fig. 7-8, Table 2-3). Cell spheroids were generated with a size below 150 μm (Fig. 3) limited by the interval of ePTFE fibrils to avoid the degeneration of inner cells because of the diffusion gradient of nutrients across the spheroids [35,36]. In addition, massive production of spheroids were visible as early as on day 1, instead of a couple of days needed in other 3D cultures [37], such as the scaffolds made of biomaterials such as alginate [38] or alginate/galactosylated chitosan hybrid polymer [39]. More potential on evaluation of hepatocytes functions exists with 3D culture with ePTFE membranes that possess a more tissue-like cultured microenvironment and thus high liver-specific functions.

In contrast to the results in sandwich culture, however, cells cultured in 3D still exhibited slightly lowered functionalities and lost their viability and biological functions at a faster rate. Sandwich culture using Matrigel as a matrix could prevent the cell loss by entrapping the hepatocytes in a 3D microenvironment as that in the liver [40,41], as clearly seen when compared with 3D (Fig. 4). Therefore, the marked enhancement and its long-term maintenance of hepatocyte functions in sandwich culture can be mainly attributed to the good

cellular preservation of hepatocytes number itself.

Although the *in vitro* microenvironment of rat hepatocytes in both sandwich culture and 3D culture were improved with regard to sufficient oxygen supply, maintaining *in vitro* engineered hepatic tissue that have *in vivo*-like functions seems to be still a challenge. As examined by PCR arrays, the gene expression levels of various drug metabolizing enzymes were considerably reduced when compared with those of freshly isolated hepatocytes (Supplement, Table 1). Further research on overall optimizing the *in vitro* microenvironments in our culture models will help to develop the fully functional rat hepatocytes *in vitro*. Also, the application of such culture models on human hepatocytes with respect to more functional assessment, especially the stability of mRNAs, is highly expected as a next step.

5. Conclusions

This study evaluated the effect of direct oxygenation through PDMS membranes on liver-specific metabolites in two promising culture models, sandwich culture and 3D culture. The present data clearly indicate that direct oxygenation through PDMS membranes considerably enhanced hepatocytes duration and metabolic activities in both cultures. As the cellular maintenance has been greatly improved by ePTFE membranes, the hepatocytes in 3D culture performed increased functionalities that comparable to those in sandwich culture.

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Tables Captions

Table 1. Parameters used in the one-dimensional diffusion reaction equation.

Table 2. Gene expression levels that changed greater to 2-fold in the PDMS-O₂ (-) cultures and the PDMS-O₂ (+) cultures compared with those in the TCPS-O₂ (-) culture on day 2.

Table 3. The number of genes those expression levels changed less than 4-fold in the TCPS-O₂ (-) culture, the PDMS-O₂ (-) cultures and the PDMS-O₂ (+) cultures compared with those of freshly isolated hepatocytes on day 2.

Figure Captions:

Fig. 1. (A) The PDMS plate with a front view (left) and a back view (right). (B) A schematic of the monolayer culture, sandwich culture with Matrigel and 3D culture with ePTFE membranes on the PDMS membranes (PDMS-O₂ (+)) or on the TCPS plates with PDMS membranes as the surface (PDMS-O₂ (-)). Conventional monolayer culture on collagen coated TCPS plates was employed as a control (TCPS-O₂ (-)). (C) Scanning electron micrograph of ePTFE. Scale bars = 20 μ m.

Fig. 2. Morphology of the hepatocytes grown in the TCPS-O₂ (-) culture, the PDMS-O₂ (-) cultures and the PDMS-O₂ (+) cultures on day 1 (A) and day 14 (B). Scale bars = 100 μ m.

Fig. 3. Photographs of hematoxylin and eosin (HE) staining of vertical thin sections of 3D cultures on Day 1 (A) and day 14 (B). Scale bars = 50 μ m.

Fig. 4. Cellular DNA amounts of attached hepatocytes on days 1 and day 14 in the TCPS-O₂ (-) culture, the PDMS-O₂ (-) cultures and the PDMS-O₂ (+) cultures. Data represent the means $\pm$ SD (n = 6) from two independent experiments. Differences with P < 0.01 (**) or P < 0.05 (*) were considered highly significant and significant, respectively.

Fig. 5. Oxygen profiles in the TCPS-O₂ (-) culture, the PDMS-O₂ (-) cultures and the PDMS-O₂ (+) cultures on day 2.

Fig. 6. Oxygen consumption rates (OCR) of the hepatocytes in the TCPS-O₂ (-) culture, the PDMS-O₂ (-) cultures and the PDMS-O₂ (+) cultures on day 2. Data represent the means $\pm$ SD (n = 3).

Fig. 7. Time course changes of albumin production in the TCPS-O₂ (-) culture, the PDMS-O₂ (-) cultures and the PDMS-O₂ (+) cultures, normalized to the DNA amounts on day 1.

Data represent the means $\pm$ SD ($n = 6$) from two independent experiments. Differences with $P < 0.01$ (**) or $P < 0.05$ (*) were considered highly significant and significant, respectively.

Fig. 8. The 7-ethoxyresorufin-O-deethylase (EROD) cytochrome P450 activity of hepatocytes cultured in the TCPS-O₂ (-), the PDMS-O₂ (-) and the PDMS-O₂ (+) on day 2 (A), day 8 (B) and day 14 (C), normalized to the DNA amounts on day 1. Data represent the means $\pm$ SD ($n = 6$) from two independent experiments. Differences with $P < 0.01$ (**) and $P < 0.05$ (*) were considered highly significant and significant, respectively.

Table 1. Parameters used in the one-dimensional diffusion reaction equation

	Parameter	Value (unit)
Culture medium	D	$2 \times 10^{-5} \text{ cm}^2/\text{s}$
	C	$760 \text{ mmHg} \times \text{ambient O}_2\%$
	K	$1.19 \times 10^3 \text{ pmol/ml/mmHg}$
	Z	0.25 cm
PDMS membrane	D	$4.1 \times 10^{-5} \text{ cm}^2/\text{s}$
	C	$760 \text{ mmHg} \times \text{ambient O}_2\%$
	K	$10.6 \times 10^3 \text{ pmol/cm}^3/\text{mmHg}$
	Z	0.05 cm
Cellular layer	C_{cell}	$760 \text{ mmHg} \times \text{measured O}_2\%$
	a	$1 \times 10^5 \text{ cells/cm}^2$

Table 2. Gene expression levels that changed greater to 2-fold in the PDMS-O₂ (-) cultures and the PDMS-O₂ (+) cultures compared with those in the TCPS-O₂ (-) culture on day 2 ^a

Gene Name	Monolayer-O ₂ (-)	Sandwich-O ₂ (-)	3D-O ₂ (-)	Monolayer-O ₂ (+)	Sandwich-O ₂ (+)	3D-O ₂ (+)
Total: 73/84	42/84	55/84	54/84	59/84	63/84	62/84
Drug Transporters:						
(6/6)	4/6	3/6	4/6	4/6	3/6	2/6
Abcb1a	-4.8 ± 0.7	-5.4 ± 1.7	-5.0 ± 0.4	-	-	-
Abcb1b	-5.5 ± 1.4	-5.8 ± 0.8	-10.1 ± 1.2	5.5 ± 1.3	3.8 ± 0.4	4.0 ± 0.8
Abcb4	-	-	-	2.5 ± 0.5	3.4 ± 0.5	3.6 ± 0.4
Abcc1	-4.3 ± 0.2	-3.9 ± 1.3	-2.8 ± 0.3	-2.2 ± 1.4	-2.5 ± 1.2	-
Mt3	-	-	-	-	-	-
Gpi	3.3 ± 0.4	-	3.8 ± 0.7	-3.2 ± 1.0	-	-
Phase I Metabolizing Enzymes:						
(14/14)	9/14	12/14	13/14	13/14	13/14	13/14
Cyp1a1	8.6 ± 1.0	10.0 ± 1.5	7.6 ± 1.1	10.8 ± 1.4	13.5 ± 2.2	11.6 ± 1.9
Cyp1a2	4.2 ± 1.0	4.1 ± 2.4	4.6 ± 2.2	6.2 ± 2.1	9.0 ± 1.2	6.9 ± 2.4
Cyp3a23/3a1	-4.2 ± 1.0	-	-3.1 ± 0.7	-2.9 ± 0.5	3.1 ± 1.7	2.4 ± 0.4
Cyp17a1	3.3 ± 1.1	3.9 ± 0.8	3.9 ± 0.6	4.2 ± 0.9	-3.3 ± 1.9	-4.0 ± 0.4
Cyp19a1	-	2.4 ± 0.4	-	2.8 ± 0.4	3.1 ± 0.4	2.4 ± 0.4
Cyp1b1	4.3 ± 1.7	-	4.3 ± 0.8	-	-	-
Cyp2b3	-	2.7 ± 0.5	3.3 ± 0.9	3.1 ± 0.7	5.8 ± 0.7	6.3 ± 0.7
Cyp2b15	-	2.5 ± 1.2	3.0 ± 1.8	2.7 ± 1.7	3.1 ± 1.7	3.6 ± 0.4
Cyp4b1	2.4 ± 0.9	3.1 ± 0.9	3.1 ± 1.4	3.9 ± 0.6	4.9 ± 1.1	4.4 ± 0.7
Cyp27b1	-	3.1 ± 1.4	-4.2 ± 0.8	3.4 ± 0.3	3.7 ± 1.1	4.1 ± 0.6
Cyp2c6v1	-	4.4 ± 2.9	13.5 ± 2.8	10.8 ± 1.8	15.2 ± 3.2	17.0 ± 2.3
Cyp2c7	3.0 ± 1.4	3.2 ± 0.7	4.7 ± 1.0	-9.9 ± 2.1	-6.4 ± 1.2	-5.4 ± 1.0
Cyp2c13	3.0 ± 0.5	4.1 ± 1.3	2.7 ± 1.1	5.7 ± 1.3	7.1 ± 2.9	6.6 ± 1.4
Cyp2e1	3.9 ± 1.5	5.1 ± 1.0	4.3 ± 1.3	5.2 ± 0.6	7.9 ± 1.7	11.3 ± 2.2
Phase II Metabolizing Enzymes:						
(45/56)	27/56	40/56	33/56	35/56	41/56	42/56
Adh1	-	-	3.1 ± 1.1	-6.9 ± 1.7	-2.7 ± 0.6	-4.1 ± 1.8
Adh4	-	-3.8 ± 1.5	-4.5 ± 1.1	3.9 ± 1.8	4.3 ± 0.4	3.4 ± 0.4
Alad	2.7 ± 1.3	-	3.3 ± 0.4	-3.4 ± 0.9	2.9 ± 0.3	2.9 ± 0.4
Aldh1a1	6.5 ± 1.5	-5.6 ± 1.8	3.4 ± 1.1	2.2 ± 0.8	-	3.0 ± 0.6
Alox15	6.4 ± 1.4	-2.7 ± 1.5	-	-	-3.5 ± 0.4	-3.7 ± 0.2
Alox5	-	-4.0 ± 1.5	-	-2.3 ± 0.9	-2.9 ± 1.1	-3.1 ± 2.2
Apoe	2.2 ± 1.1	3.3 ± 1.2	3.5 ± 0.7	-	3.6 ± 0.9	3.6 ± 0.6
Blvrb	-2.5 ± 0.8	-4.6 ± 0.8	-	-4.0 ± 0.7	-5.3 ± 1.0	-5.6 ± 0.4
Ces1e	3.5 ± 0.7	4.5 ± 0.8	7.8 ± 0.2	6.1 ± 0.9	9.7 ± 0.6	14.0 ± 6.8
Ces2c	-	4.0 ± 1.7	2.9 ± 0.4	2.4 ± 0.9	6.8 ± 1.8	3.3 ± 1.0
Chst1	2.4 ± 0.7	3.2 ± 1.4	2.9 ± 0.8	4.9 ± 1.4	4.9 ± 0.7	6.2 ± 1.3
Comt	-	3.5 ± 2.0	3.4 ± 1.6	-	2.9 ± 0.8	2.7 ± 1.0
Ephx1	2.5 ± 1.2	3.4 ± 1.2	5.5 ± 3.7	5.8 ± 1.2	6.4 ± 1.4	7.3 ± 1.1
Faah	4.2 ± 1.1	3.4 ± 1.0	5.6 ± 1.6	5.9 ± 1.6	6.5 ± 3.2	7.6 ± 2.3
Fbp1	3.8 ± 0.8	5.4 ± 1.1	4.3 ± 1.2	4.2 ± 1.4	7.0 ± 1.8	5.5 ± 0.6
Gad1	3.8 ± 2.1	-	4.6 ± 1.3	-2.8 ± 0.4	-3.5 ± 1.3	-2.3 ± 1.2
Gad2	2.1 ± 0.7	2.6 ± 0.6	3.2 ± 0.7	2.4 ± 0.2	3.5 ± 1.0	4.0 ± 0.4
Ggt1	3.5 ± 1.6	4.9 ± 2.4	8.0 ± 1.6	-	-	-3.7 ± 1.4
Gpx2	-7.3 ± 3.0	-5.2 ± 1.0	-	-5.6 ± 1.8	-4.6 ± 1.4	-5.0 ± 1.2
Gpx3	-	4.4 ± 0.9	3.9 ± 1.3	5.5 ± 1.3	6.0 ± 1.9	5.6 ± 0.7

Gpx5	-	-	-	-	2.4 ± 1.2	2.2 ± 1.2
Gsr	-3.8 ± 1.2	-2.3 ± 1.4	-	-	2.3 ± 0.9	2.4 ± 0.3
Gsta1	-	-	-	2.4 ± 1.1	3.2 ± 0.3	3.0 ± 0.9
Gsta4	5.0 ± 0.9	5.9 ± 1.0	7.8 ± 2.8	9.9 ± 1.6	10.9 ± 1.2	12.3 ± 1.1
Gstm1	-2.1 ± 1.4	-	2.8 ± 1.4	-	3.5 ± 0.8	4.0 ± 1.3
Gstm2	-	-	-	3.0 ± 0.9	3.3 ± 1.3	3.0 ± 1.0
Gstm3	-	2.7 ± 1.7	3.0 ± 0.9	2.9 ± 0.3	8.5 ± 0.7	6.7 ± 3.1
Gstm4	-	-	-	-	2.4 ± 0.6	-
Gstm5	-	-	-	2.8 ± 1.6	3.0 ± 1.5	2.8 ± 1.1
Gstp1	-4.0 ± 0.8	-4.7 ± 1.2	-6.2 ± 1.1	-4.5 ± 1.2	-7.0 ± 2.6	-7.2 ± 1.6
Gstt1	-	2.3 ± 1.1	3.2 ± 1.4	3.0 ± 1.1	3.6 ± 0.6	3.4 ± 0.6
Hk2	2.63 ± 1.3	2.9 ± 1.1	2.8 ± 0.8	4.1 ± 1.1	4.2 ± 1.0	4.2 ± 1.0
Hsd17b1	-2.5 ± 1.4	-2.1 ± 0.2	-	-	2.5 ± 0.8	3.0 ± 0.9
Hsd17b2	8.2 ± 2.5	10.3 ± 2.2	9.3 ± 2.1	12.1 ± 1.7	14.0 ± 1.2	13.2 ± 1.3
Mgst3	-	2.1 ± 0.7	2.3 ± 0.1	2.3 ± 0.1	2.8 ± 0.6	2.5 ± 0.6
Nat1	2.6 ± 0.2	2.7 ± 1.5	2.8 ± 0.7	2.7 ± 1.1	3.2 ± 1.4	2.9 ± 0.6
Nos2	-	-	2.3 ± 1.3	2.6 ± 0.5	4.3 ± 1.6	-
Nos3	3.2 ± 1.6	4.0 ± 0.8	3.4 ± 0.8	4.4 ± 0.7	5.7 ± 1.0	5.0 ± 2.0
Nqo1	-3.0 ± 2.0	-3.6 ± 2.1	-2.9 ± 0.7	-2.3 ± 0.6	-	2.2 ± 0.9
Pklr	3.2 ± 2.2	7.2 ± 1.8	8.2 ± 2.0	3.0 ± 1.7	6.1 ± 2.8	3.3 ± 0.7
Pkm	3.8 ± 1.0	5.2 ± 1.2	3.9 ± 1.0	-3.3 ± 1.0	-4.0 ± 1.1	-4.0 ± 1.3
Pon2	-	3.0 ± 1.4	4.3 ± 2.1	4.2 ± 2.2	2.1 ± 0.7	3.1 ± 1.1
Pon3	-	3.1 ± 1.1	3.0 ± 0.8	2.9 ± 0.6	4.4 ± 1.2	3.9 ± 1.1
Srd5a1	2.5 ± 1.7	3.8 ± 0.9	2.9 ± 1.4	-9.4 ± 1.9	-7.0 ± 1.9	-8.5 ± 2.2
Other Genes:						
(8/8)	2/8	6/8	4/8	7/8	6/8	5/8
Aoc1	-	-	-	2.2 ± 0.5	2.3 ± 0.9	2.3 ± 0.5
Ahr	-	-3.4 ± 1.4	3.0 ± 1.8	-2.8 ± 1.3	-2.2 ± 1.1	-
Arnt	-	2.127 ± 0.2	2.4 ± 1.2	2.2 ± 1.6	2.8 ± 0.6	3.0 ± 0.5
Asna1	-	-3.1 ± 2.1	-	-	-	-
Gckr	3.2 ± 1.0	3.2 ± 1.1	4.0 ± 1.6	3.5 ± 0.7	4.7 ± 0.7	3.8 ± 1.0
Marcks	-	-	-	2.8 ± 0.9	3.1 ± 1.4	3.5 ± 1.0
Smarcal1	-	-2.1 ± 0.7	-	-2.6 ± 1.6	-	-
Snn	3.8 ± 1.7	2.9 ± 1.0	3.8 ± 1.9	3.4 ± 1.8	2.4 ± 0.5	2.6 ± 1.4

^a The gene expression levels were determined on day 2 using an RT² profiler. The gene expression levels were normalized to 1 in the TCPS-O₂ (-) culture. PCR array: rat drug-metabolism genes, including drug transporters, phase I metabolizing enzymes, phase II metabolizing enzymes and several other genes. Data represent the means ± SD (n = 3).

Table 3. The number of genes those expression levels changed less than Gene-expression levels that changed up to 4-fold in the TCPS-O₂ (-) culture, the PDMS-O₂ (-) cultures and the PDMS-O₂ (+) cultures compared with those of freshly isolated hepatocytes on day 2 ^a

	TCPS- O ₂ (-)	Monolayer- O ₂ (-)	Sandwich- O ₂ (-)	3D- O ₂ (-)	Monolayer- O ₂ (+)	Sandwich- O ₂ (+)	3D- O ₂ (+)
Total	28	34	39	38	44	48	47
Drug transporter	2	3	2	3	3	3	3
Phase I Metabolizing Enzymes	1	1	2	1	3	4	4
Phase II Metabolizing Enzymes	22	26	30	29	32	35	34
Other genes	3	4	5	5	6	6	6

^a The gene expression levels were determined on day 2 using an RT² profiler. PCR array: rat drug-metabolism genes, including drug transporters, phase I metabolizing enzymes, phase II metabolizing enzymes and several other genes.

A

Fig. 1

B

TCPS-O₂ (-)

PDMS-O₂ (-)

PDMS-O₂ (+)

Fig. 1

C

Fig. 1

A

TCPS-O₂ (-)

Monolayer

Sandwich

3D

PDMS-O₂ (-)

PDMS-O₂ (+)

Fig. 2

B

TCPS-O₂ (-)

Monolayer

Sandwich

3D

PDMS-O₂ (-)

PDMS-O₂ (+)

Fig. 2

A**3D-O₂ (-)** **3D-O₂ (+)** **B****3D-O₂ (-)** **3D-O₂ (+)** **Fig. 3**

Fig. 4

A **B** **C** **Fig. 5**

Fig. 6

Fig. 7

A **B** **C** **Fig. 8**