論 文 の 内 容 の 要 旨

論文題目 Development of hepatocyte culture systems for drug screening through optimization of culture microenvironments
（培養微小環境の最適化を通じた創薬スクリーニング用肝細胞培養系の開発）

氏　　名　肖 文晋

Chapter 1. General introduction

As the largest visceral in human, the liver acts as the center of metabolism and is one of the most important sites of drug ADME (absorption, distribution, metabolism and excretion)-Efficiency/Toxicity. Since the majority of liver functions, including xenobiotic biotransformation, is mainly performed by hepatocytes, hepatocyte-based *in vitro* models are currently being applied in drug development. Consequently, the development, characterization and validation of long-term cultures of hepatocytes as *in vitro* experimental models represent an important goal in experimental and regulatory toxicology, drug development and hazard identification/evaluation. However, the *in vitro* maintenance of functional primary hepatocytes has been a challenge, as isolated hepatocytes rapidly lose many of their morphological and functional characteristics in the conventional culture models. To overcome these problems, many studies have investigated improvements in the culture environments.

Two widely accepted hepatocytes culture models have been developed, sandwich culture and 3D culture. Sandwich culture, in which the cells are entrapped between two gel layers of collagen, is a well established cellular model for the hepatocytes considering the restoration of cell-extracellular matrix (ECM) contacts, one of the key roles in the culture configurations of primary hepatocyte. However, to access to the hepatocytes for metabolic and toxicity analysis, the first layer of collagen gel has to be enzymatically digested. To solve this problem, an overlay of Matrigel, a basement membrane extract from mouse sarcoma, has been used to simplify the culture model by soaking the hepatocyte layer in Matrigel-containing culture medium. The other advanced format, in which the hepatocytes could be cultured in a 3D configuration to represent the cell-to-cell contacts, has also been developed of late years. In 3D culture, the hepatocytes maintain not only *in vivo*-like morphological characteristics, but also liver-specific functionalities. However, the decreased functionalities in 3D culture were previously found, as a result of the deficient cellular maintenance and the degeneration of inner cells due to oxygen shortage. Expanded polytetrafluoroethylene (ePTFE), a macroporous synthetic polymer, has thus been developed as one of the preferable substrates to improve stability in culture, according to firmly attached hemispheroids. Thus, an improved long-term culture model is highly expected by quantitative comparison of hepatocyte functions between sandwich culture and 3D culture with advanced substrates.

Oxygen is one of the vital environmental 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. 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, forcing the hepatocytes to undergo hypoxia. Besides, current cell cultivation is mainly maintained under atmospheric oxygen levels (20% O_2), several fold higher than the values those cells encounter under physiological conditions *in vivo*. In the liver lobule, the concentration of oxygen decreases from 13% (arterial) to 9-11% (periportal) and then to 4-6% (hepatovenous) from the periportal to the perivenous area. These data suggest that culturing hepatocytes under physiological oxygen concentrations may prolong cell survival and reduce the rates of decline in hepatic functions. In our previous work, to solve these problems, we have developed a simple method of culturing cells on an oxygen-permeable membrane, polydimethylsiloxane (PDMS), to meet these intense requirements for oxygen. As governed by Henry's law, the actual oxygen concentration at the cell level could be very close to the ambient concentrations. The PDMS membranes could facilitate direct oxygenation by diffusion through the membranes, which is expected to completely meet the cellular OCR requirements.

The hepatic microenvironment is a complex structure comprising of multiple cell types. There is a growing recognition that hepatic cell cultures should mimic the *in vivo* structure of the liver, in which hepatocytes are in contact with non-parenchymal cells via the Space of Disse. The most successful co-culture system used as helper cells was obtained with liver sinusoidal endothelial cells (LSECs), which account for more than 80% of the liver's mass together with hepatocytes. However, it is still difficult to realize the *in vivo*-like double layered cyto-architecture constituting of hepatocytes and endothelial cells. Our previous work has shown that a multilayer structure was successfully achieved by co-culturing hepatocytes with epithelial cells on the PDMS membranes. Therefore, it is highly expected that the hepatocytes co-cultured with endothelial cells on the PDMS membranes may recover a complete double layered configuration, and also retain to greater extent morphological and biochemical characteristics of hepatocytes.

To sum up, based on the direct oxygenation through the PDMS membranes, the objective of this dissertation is to develop a hepatocyte culture system through optimization of culture microenvironments for drug screening. A long-term hepatocytes culture model was firstly established by comparing sandwich culture with 3D culture using advanced substrates (Chapter 2). Major issues of the microenvironments were next optimized in this culture model, including oxygen concentrations (Chapter 3) and co-culturing with endothelial cells (Chapter 4), to maintain high metabolic capacities of hepatocytes in a long duration.

Chapter 2. Effect of direct oxygen supply through the PDMS membranes on hepatocytes in two best models: comparison of sandwich culture with 3D culture

In this chapter, I aimed to explore an improved hepatocytes culture model with sufficient oxygen supply. The effect of direct oxygenation using PDMS membranes on the functionalities of hepatocytes in both sandwich culture with Matrigel and 3D culture with ePTFE membranes was investigated and compared. Rat hepatocytes were cultured on the PDMS membranes in monolayer culture, sandwich culture with Matrigel and 3D culture with ePTFE membranes for 14 days in the presence and absence of direct oxygenation from the other side of the membranes [PDMS-O_2 (-), PDMS-O_2 (+)]. Conventional monolayer culture on the TCPS plates was employed as a control [TCPS-O_2 (-)]. The present results showed remarkable enhancement of hepatocytes duration and functions by oxygen diffusion through the PDMS membranes. Cells formed a confluent monolayer in both Monolayer-O_2 (+) and Sandwich-O_2 (+) cultures, and spheroids in 3D-O_2 (+) culture from day 1 and maintained large attachment on day 14 (Fig. 1-1). The oxygen concentrations at the cell layer accurately presented the ambient levels in the PDMS-O_2 (+) cultures (Fig. 1-2), allowing the OCR values in the normoxic range (Fig. 1-3). Whereas, oxygen concentrations valued half in the TCPS-O_2 (-) culture and the PDMS-O_2 (-) cultures, with the OCR values indicating that cells were in extremely hypoxia. In addition, the hepatocytes cultured in Sandwich O_2 (+) exhibited highest maintenance of metabolic activities, such as albumin production (Fig. 1-4) and Cyp1a1/2 activity (Fig. 1-5). This study clearly illustrated that oxygenation is a critical factor to be considered in optimization of the microenvironments of hepatocyte cultures, and Sandwich-O_2 (+) culture with Matrigel is a superior culture model when comparing with 3D culture in terms of cellular preservation.

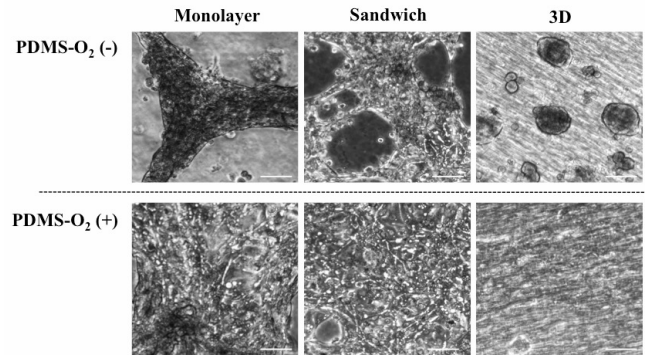

Fig. 1-1 Morphology of hepatocytes on day 14. Scale bars=100μm.

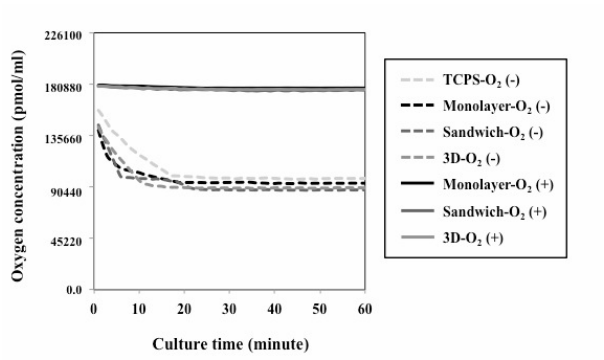

Fig. 1-2 Oxygen concentration at the cell level on day 2.

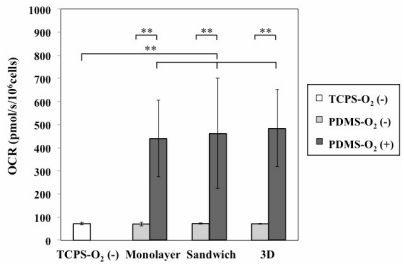

Fig. 1-3 Oxygen consumption rate (OCR) on. day 2. Data represent the means ± SD (n=3).

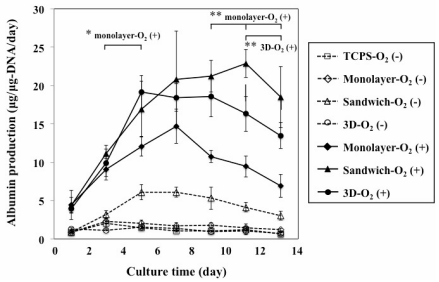

Fig. 1-4 Time course of albumin production. Data represent the means ± SD (n=6).

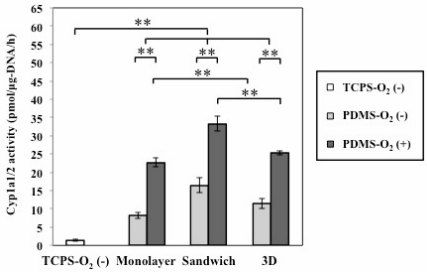

Fig. 1-5 Cyp1a1/2 activity on day 14. Data represent the means ± SD (n=6).

Chapter 3. Optimization of oxygen concentration in sandwich culture with direct oxygenation through the PDMS membranes

I next optimized the oxygen concentrations at the cell level. Upon the results of Chapter 2, I proposed in this chapter a PDMS membranes-based sandwich culture, where the cellular OCR requirements can be completely meet. Various functional characteristics of the hepatocytes cultured under either atmospheric oxygen tension (20%) or sub-physiological conditions (10% and 5%) were systematically evaluated and compared for simulating the *in vivo* average oxygen levels. In parallel, the PDMS-O_2 (-) culture was carried out as a control group (20%-O_2 (-)). Morphology showed 14 days culture was successfully achieved under 10%-O_2 (+). On day 2, the oxygen concentration was maintained as the ambient oxygen levels under 20%-O_2 (+), 10%-O_2 (+) and 5%-O_2 (+) (Fig. 2-1). The normoxic OCR values of 380-700 pmol/s/10^6 cells demonstrated that the oxygen concentrations exceeding 10% in the PDMS-O_2 (+) cultures were sufficient to sustain cellular aerobic metabolism (Fig. 2-2). The increased Cyp1a1/2 activity (Fig. 2-3) and accumulation of bile canaliculi formation (Fig. 2-4) under 10%-O_2 (+) also supported the view that 10%-O_2 (+) helped to evaluate *in vitro* feasibility functional zonation in lobules. These results indicated that it is important to maintain cellular oxygen concentration at *in vivo*-like physiological levels and 10%-O_2 (+) contributed to prolong the cell survival and enhance metabolic functions of primary hepatocytes.

Chapter 4. Further improvement of culture microenvironment: co-culture with non-parenchymal cells

In this chapter, I proposed a co-culture of hepatocytes overlaid with TMNK-1 to form a sandwich configuration on the PDMS membranes to enable a double layered liver tissue. One main benefit of such co-cultivation technique on metabolic competence of hepatocytes is the excreted ECM to replace Matrigel in the sandwich culture of Chapter 2. Based on the proper ratio of seeding density between hepatocytes and TMNK-1 (H:T=2:1), various functional characteristics of the hepatocytes cultured in co-culture were systematically evaluated and compared with those in sandwich culture with Matrigel. After 1 day inoculation of hepatocytes in Monolayer-O_2 (+), TMNK-1 cells were seeded overlay the confluent hepatocytes monolayer. Hematoxylin and eosin (HE) staining clearly showed double layered configuration in the case of Coculture-O_2 (+) (Fig. 3-1), with TMNK-1 being easily recognizable with their flat morphology contrasting with the larger and cuboidal morphology of hepatocytes. The result of hepatic functions, such as increased Cyp1a1/2 activity (Fig. 3-2), supported that TMNK-1 helped hepatocytes to display higher metabolic activities

with better maintenance than those in sandwich culture. We concluded that by co-culturing hepatocytes with TMNK-1 on the PDMS membranes, a double layered architecture of functional liver cells was successfully obtained, which could potentially enhance liver specific behavior of hepatocytes.

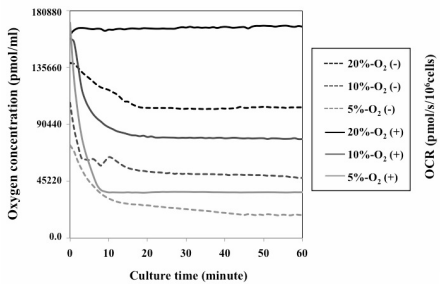

Fig. 2-1 Oxygen concentration at the cell level on day 2.

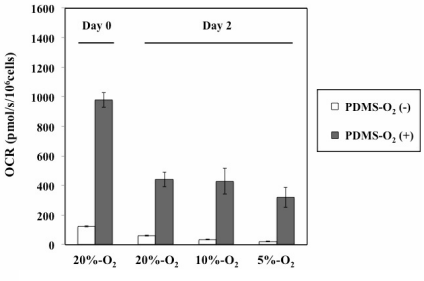

Fig. 2-2 Oxygen consumption rate (OCR). Data represent the means ± SD (n=3)

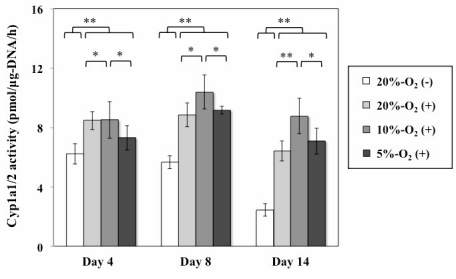

Fig. 2-3 Cyp1a1/2 activity on day 4, day 8, and day 14. Data represent the means ± SD (n=6).

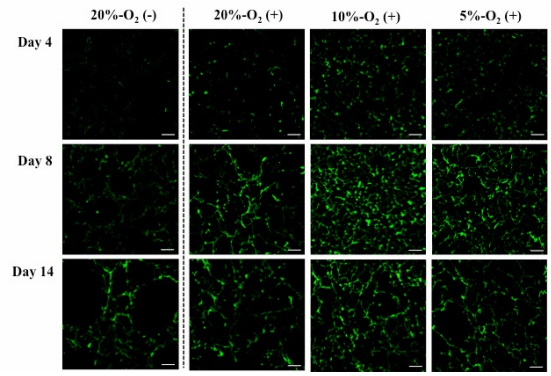

Fig. 2-4 Analysis of CDFDA on day 4, day 8 and day 14. Scale bars =100 μm.

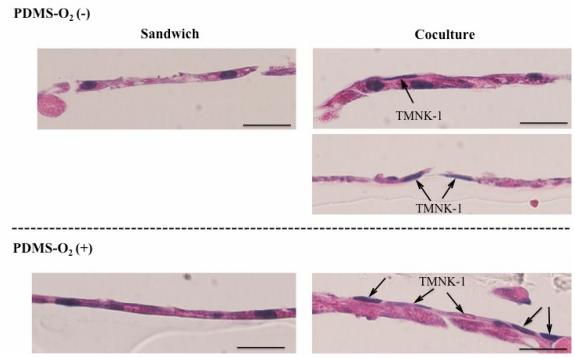

Fig. 3-1 Hematoxylin and eosin (HE) staining on day 4. Scale bars=50μm.

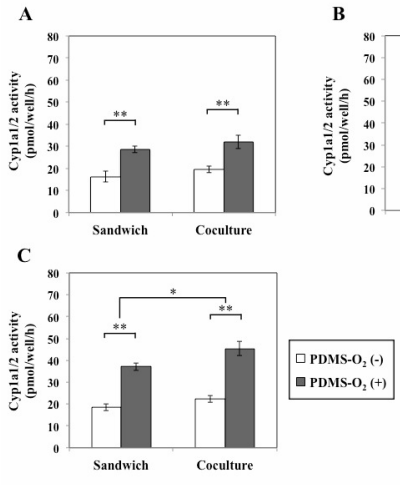

Fig. 3-2 Cyp1a1/2 activity on day 4 (A), day 8 (B) and day 14 (c). Data represent the means ± SD (n=3).

Chapter 5. General conclusion and future perspectives

I summarize the results here: 1. Direct oxygenation through the PDMS membranes resolved the oxygen shortage in the conventional culture of hepatocytes, and sandwich culture with Matrigel on the PDMS membranes served as a superior culture model; 2. It is important to satisfy cellular oxygen demand at *in vivo*-like physiological levels: 10%-O_2 (+) helped to extend the hepatocytes survival and enhance metabolic activities for at least 14 days; 3. Co-culturing hepatocytes with TMNK-1 enabled double layered functional liver cells mimicking *in vivo* liver cytoarchitecture. In conclusion, a novel culture of hepatocytes was appealed in this dissertation through improving simultaneously culture model and microenvironments. The integrated approach provides the hepatocytes with better-maintained duration and hepatic functionalities.

For the future perspective, we are considering further application of this culture model on human hepatocytes through improvement of culture microenvironments, with regard to appropriate oxygen concentration on co-cultured liver cells. To replace the source of primary hepatocytes from the liver, hepatic induction of various stem cells based on iPSCs technologies will be developed and applied in the optimized culture to reconstruct the multilayer 3D liver issue with high metabolic capacities, which will be used for assessing hepatitis virus and also toxicity studies.