Fundamental Toxicological Sciences (Fundam. Toxicol. Sci.)
Vol.11, No.1, 17-25, 2024

The Japanese Society of Toxicology

Fundamental Toxicological Sciences
URL : http://www.fundtoxicolsci.org/index_e.html

Original Article

Comparative analysis of bile canaliculi formation in fresh and flask-delivered human hepatocytes from humanized mouse livers under sufficient oxygen supply

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(Received January 30, 2024; Accepted February 8, 2024)

ABSTRACT — Functional bile canaliculus formation in cultured human hepatocytes is crucial for *in vitro* studies of hepatobiliary disposition and drug-induced cholestasis. Human hepatocytes isolated from humanized mouse livers (PXB-cells) are promising cell sources for these studies. PXB-cells available in tissue culture flasks allow users to recover and reseed in different cell culture formats, thereby enhancing their adaptability to various *in vitro* culture systems. However, the reseeding process may induce cellular stress, affecting subsequent cultures, and its specific effect on bile canaliculus formation is yet to be explored. Furthermore, the role of sufficient oxygen supply in bile canaliculus formation in PXB-cells remains incompletely understood. In this study, we compared hepatic function and bile canaliculi formation in freshly seeded PXB-cells (Fresh PXB-cells) and reseeded PXB-cells (Flask-delivered PXB-cells) under sufficient oxygen supply through oxygen-permeable plates. The flask-delivered PXB-cells recovered their levels of albumin production and cytochrome gene expression to those of fresh PXB-cells after seven days of culture. On days seven and 14 of culture, bile canaliculus formation was similar in both fresh and flask-delivered PXB-cells, as confirmed by fluorescein-labeled bile acid excretion and immunostaining for the bile canaliculi marker MRP2. Notably, analysis of bile canalicular length revealed a significant increase in bile canalicular length with adequate oxygenation, whereas no significant difference was detected between the conditions under the same oxygen supply on days seven and 14. The findings of this study provide valuable insights into the use of PXB-cells for *in vitro* assessments in drug discovery and toxicological research.

Key words: PXB-cells, Hepatocytes, Sandwich culture, Bile excretion, Bile canaliculi, Oxygen

INTRODUCTION

Understanding and predicting human biliary excretion are paramount for studies on hepatobiliary disposition and drug-induced liver injury. Biliary excretion is an important pathway for the release of drugs, chemicals, and metabolites from the liver. In some cases, compounds excreted into bile may undergo reabsorption by the intestine and return to the liver through enterohepatic circulation (Roberts *et al*., 2002). This circulation potential-

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ly prolongs the duration of action of these compounds, thereby amplifying their pharmacological effects and inducing organ toxicity. Additionally, certain pharmaceutical compounds can disrupt bile acid excretion by interacting with bile transporters, leading to cholestasis and, eventually, liver injury (Susukida *et al*., 2015). Therefore, an accurate assessment of biliary excretion is essential to comprehensively understand and ensure the safety of drug compounds.

The current approach to *in vitro* analysis of human biliary excretion mainly relies on culturing primary human hepatocytes (PHHs) sandwiched between two layers of extracellular matrix, which can physiologically reproduce the formation of bile canaliculi between adjacent hepatocytes (Kitaguchi *et al*., 2023; Liu *et al*., 1999; Sakai *et al*., 2023). Nevertheless, because PHHs are commonly cryopreserved, the freezing/thawing process often induces cellular damage, such as the generation of excessive reactive oxygen species (Stéphenne *et al*., 2007), which hinders stable bile canalicular formation and affects the accuracy of biliary assays (Horiuchi *et al*., 2022; Kitaguchi *et al*., 2023; Sakai *et al*., 2023).

The formation of stable and high-capacity bile canaliculi is strongly desired for the accurate measurement of biliary compounds, because the accuracy of measurement relies on the amount of biliary compounds present in the canaliculi. Fresh PHHs from chimeric mice with humanized livers (PXB-cells) have emerged (Shinha *et al*., 2021), demonstrating better bile canaliculus formation compared with that of cryopreserved PHHs (Kohara *et al*., 2020). However, the bile canaliculi of PXB-cells are not as branched as those in the human liver, hampering accurate bile analysis. Studies have demonstrated the acceleration of elongated bile canaliculi formation in rat primary hepatocytes under sufficient oxygen supply via oxygen-permeable plates (Matsui *et al*., 2010), suggesting the potential enhancement of bile canaliculi formation in PXB-cells with similar oxygenation.

PXB-cells are currently available in ready-to-use conventional tissue culture multi-well plates (Fresh PXB-cells) or in tissue culture flasks (Flask-delivered PXB-cells) for reseeding to unique culture plates and devices, such as the oxygen-permeable plates mentioned previously (Fig. 1). To reseed the PXB-cells from the tissue culture flasks to other plates, trypsin was used for cell detachment. However, despite the possible alterations in cellular function following trypsin treatment (Huang *et al*., 2010; Lai *et al*., 2022), the effects of reseeding on liver function and bile canalicular formation have not been well validated.

Therefore, in this study, we compared hepatic functions and bile canaliculi formation in fresh or flask-delivered PXB-cells, while ensuring sufficient oxygen supply with oxygen-permeable plates, to investigate the effect of oxygen supply and reseeding on bile canaliculi formation. These results are important for the proper utilization of PXB-cells in drug discovery and toxicological research.

MATERIALS AND METHODS

Materials and reagents

Collagen-coated polymethyl pentene-bottomed 96-well plate, an oxygen-permeable plate, was purchased from Mitsui Chemicals Inc. (Tokyo, Japan) (Nishikawa *et al*., 2022). The plate was sealed with a polyester film (Applied Biosystems, MA, USA) to prevent oxygen supply from the bottom. Williams' medium E (Gibco, CA, USA) containing 2% Fetal Bovine Serum (FBS) (Biowest, Nuaillé, France), 1% Penicillin-Streptomycin Solution (Wako, Osaka, Japan), 20 mM 4-(2-hydroxyethyl)-1-piperazieethanesulfonic acid (HEPES) (Gibco), 0.25 µg/mL Insulin (Takara, Shiga, Japan), 50 nM Dexamethasone (Wako), 5 ng/mL human epidermal growth factor (Peprotech, NJ, USA), and 0.5 mM ascorbic acid 2-phosphate (Wako) was used as the maintenance culture for PXB-cells.

Cell culture

Human hepatocytes isolated from humanized mice with chimeric livers (PXB-cells) were obtained from Phoenix Bio (Hiroshima, Japan) (Fig. 1). Fresh-PXB-cells were seeded at a density of 2.1×10^5 cells/cm^2 onto the plate just after being isolated from the mice. For the flask-delivered PXB-cells conditions, the live cells were maintained in T-25 or T75 flasks for two days for the delivery and detached with 0.25% Trypsin-0.53 mM EDTA (Wako). Cell viability was > 80%, which was confirmed using trypan blue staining, and the live cells were seeded onto the oxygen-permeable and non-oxygen-permeable plates (hereinafter called "oxygen supply (+)" and "oxygen supply (−)," respectively). Cells were cultured at 37°C and 5% CO_2 for two weeks. Cells in oxygen supply (−) conditions were cultured in a 20% O_2 atmosphere, whereas those in oxygen supply (+) condition were cultured in a 10% O_2 atmosphere to reproduce physiological oxygen concentration (Scheidecker *et al*., 2020). The cells were supplemented with 2% v/v Matrigel (Corning, NY, USA) from day one, and the medium was changed every other day (100 µL per well in 96-well format).

Measurement of albumin production

Human albumin from samples on days three, five,

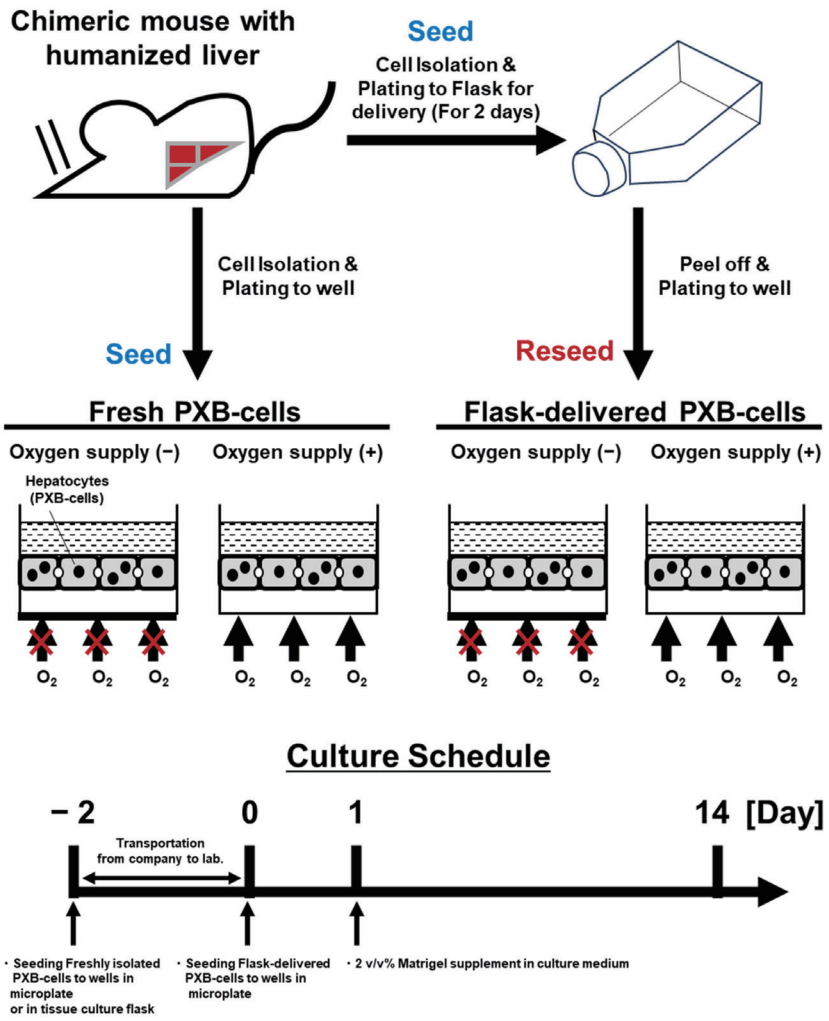

Fig. 1. The outline of this experiment. The isolated fresh human hepatocytes from chimeric mice with humanized liver were seeded into oxygen-permeable or non-oxygen-permeable multi-well plates (Fresh PXB-cells) or tissue culture flasks (Flask-delivered PXB-cells) and delivered to the laboratory for two days. PXB-cells in tissue culture flasks were harvested with 0.25% trypsin-0.53 mM EDTA and reseeded into oxygen-permeable or non-oxygen-permeable multi-well plates immediately upon arrival. Cells were cultured for 14 days and supplemented with 2% v/v Matrigel from day one.

seven, nine, 11, and 13 was measured by sandwich-type enzyme-linked immunosorbent assay, as previously described (Nishikawa *et al*., 2022). Goat anti-human albumin antibody (Bethyl Laboratories, Texas, USA) was used as the primary antibody and goat anti-human albumin conjugated with horseradish peroxidase (Bethyl Laboratories) was used as the secondary antibody. The reference standard was obtained from a human albumin solution prepared from human albumin powder (Wako). The absorbance was measured at 450 nm using a microplate reader (Bio-Rad Laboratories, CA, USA).

Observation of fluorescein-labeled bile acids excretion

On day seven and day 14, cells were exposed to 5 μM cholyl-lysyl-fluorescein (CLF) (Corning) in HBSS (+) (Wako) for 30 min at 37°C in 5% CO_2/air, followed by washing three times with cold HBSS (+). Stained cells were observed using confocal microscopy at 20 × magnification (Olympus, Tokyo, Japan).

Gene Expression Analysis by RT-qPCR

On day seven, cells were dissociated with TRIzol (Thermo Fisher Scientific, Waltham, MA, USA), and

total RNA was extracted, phase-separated with chloroform, and precipitated using isopropanol. The extracted RNA was reverse-transcribed using the PrimeScript RT Reagent Kit (Takara), and quantitative real-time polymerase chain reaction was performed using the Thunderbird SYBR qPCR reagent (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The fold change differences between samples were determined using the comparative Ct method (ΔΔCt) with β-actin as the calibrator. The data were normalized by the sample of the fresh PXB-cells in the oxygen supply (−) condition on day seven. The primer sequences were as follows: ACTB-fw, CCTCATGAAGATCCTCACCGA; ACTB-rev, TTGCCAATGGTGATGACCTGG; CYP3A4-fw, ACATAGCCCAGCAAAGAGCAAC; and CYP3A4-rev, GTCTGGGATGAGAGCCATCACT.

Immunostaining

Immunostaining was performed as described previously (Matsui *et al*., 2010). Briefly, cells on days seven and 14 were fixed with 4% paraformaldehyde (Wako) for 20 min and blocked with PBS containing 1% BSA (Wako) and 0.1% tween-20 (Wako) for 30 min at room temperature. The primary antibody for MRP2 (GeneTex, CA, USA) was applied to the fixed cells and kept at 4°C overnight. Subsequently, a secondary antibody (Abcam, Cambridge, UK) and phalloidin-iFluor 555 reagent (Abcam) were added and incubated for 1 hr at room temperature. Samples were observed by confocal microscopy at 40x magnification (Olympus).

Measurement of bile canalicular length

The bile canalicular length was measured under each condition using immunostaining images (40x magnification). as described previously (Fu *et al*., 2011; Ikebuchi *et al*., 2012). Briefly, the length of the bile canaliculi was measured based on the colocalization of MRP2 and F-actin using the cellSens software equipped with an Olympus FV3000 (Olympus). The bile canalicular length per image was summed and divided by the number of cells counted using ImageJ software to obtain the canalicular length per cell. Twelve images were randomly selected for each condition from at least two independent experiments, and measurements were performed by at least two investigators.

Statistical analysis

The results are presented as the mean ± the standard deviation as indicated in the figure legends. All experiments were independently repeated two or three times. One-way analysis of variance followed by Tukey's statistically significant difference post-hoc test was performed for multiple comparisons. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Comparison of hepatic function

The fresh and flask-delivered human hepatocytes isolated from chimeric mice (PXB-cells) were cultured in the sandwich culture configuration for 13 days in the oxygen supply (±) condition after inoculating flask-delivered PXB-cells on the plates (Fig. 1). The cell viability of the fresh PXB-cells and the flask-delivered PXB-cells at seeding was 87.4 ± 2.7 and 84.8 ± 1.1%, respectively, with no significant difference (N=3, biological replicates). Under all conditions, the PXB-cells maintained their intact cell layer until the end of the culture, as confirmed by phase contrast microscopy (Supplement S1).

We first examined the hepatic function of fresh- and flask-delivered PXB-cells under oxygen supply (±) conditions. Albumin production in fresh PXB-cells remained relatively constant until day 13, whereas that in flask-delivered PXB-cells gradually increased over time, reaching secretion levels comparable with those of fresh cultures on day seven (Fig. 2A). Notably, an adequate oxygen supply enhanced albumin production under both fresh and flask-delivered conditions. This beneficial effect of the oxygen supply was better observed in fresh PXB-cells up to day seven. In contrast, the gene expression levels of *CYP3A4*, one of the major metabolic enzymes in the liver, showed no significant differences among the different conditions on days seven and 14 (Fig. 2B).

Comparison of bile canaliculi formation

Since the results of albumin production indicated that all conditions reached functional equilibrium after seven days of culture, we examined bile canaliculi formation under all conditions on days seven and 14. Fluorescein-labeled bile acid (CLF), which is excreted into the bile canaliculi via apical transporters, was used to observe bile excretion. CLF excretion levels in the bile canaliculi of the flask-delivered PXB-cells were comparable with those of fresh PXB-cells (Fig. 3). More importantly, under adequate oxygen supply conditions, CLF excretion into bile canaliculi of both fresh- and flask-delivered PXB-cells was enhanced, and bile canaliculi formation was more extended than under oxygen supply (−) conditions. Immunofluorescence staining of F-actin, a marker of bile canaliculi architecture, and MRP2 transporters, a transporter expressed on the apical membrane, also demonstrated compatible bile canaliculi formation in flask-

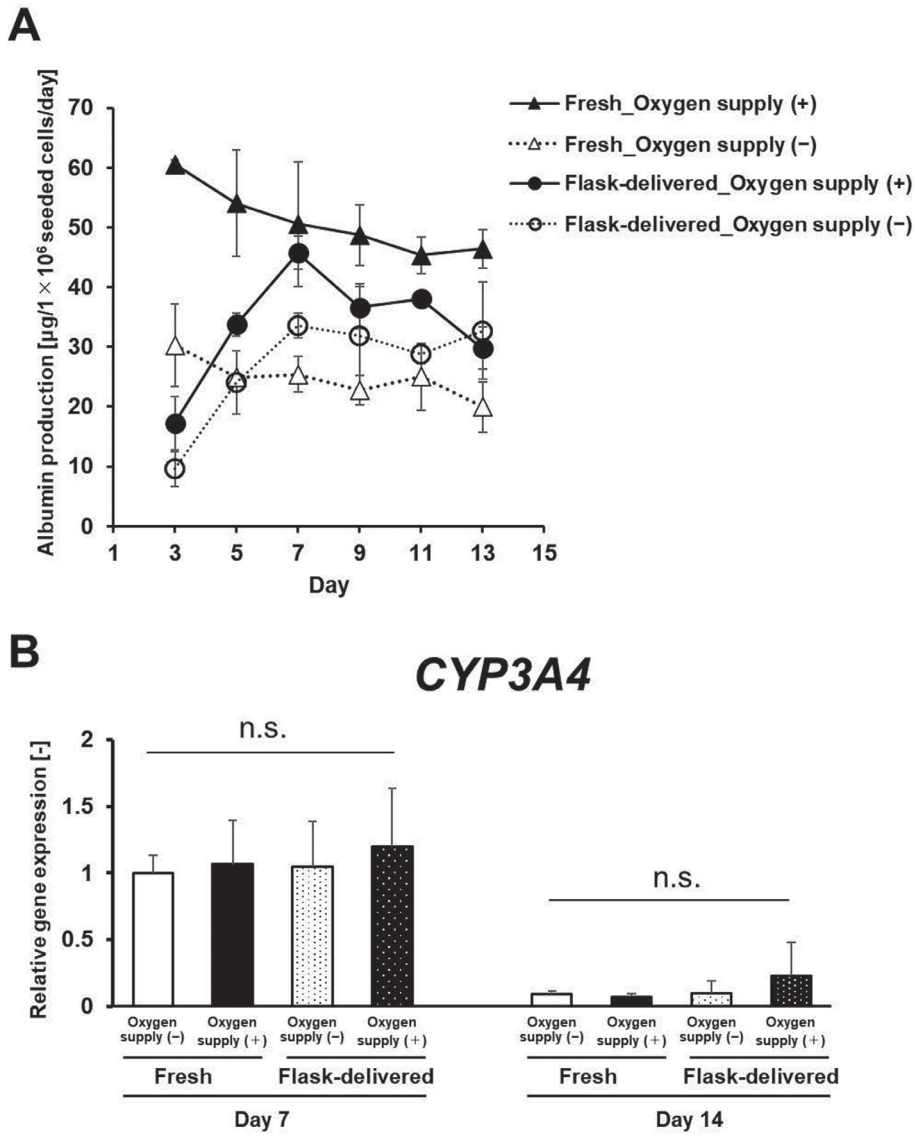

Fig. 2. Comparison of the major hepatic function. **A.** Daily albumin production in each condition. N = 3, biological replicates, mean ± standard deviation. **B.** The relative gene expression of *CYP3A4*. The data were normalized by the condition of fresh-PXB-cells in oxygen supply (−) on day seven. N = 3, biological replicates, mean ± standard deviation. n.s.: no significant difference.

delivered PXB-cells to fresh PXB-cells and enhancement of bile canaliculi formation under oxygen supply (+) conditions (Fig. 4A). These results correspond well with those of CLF excretion (Fig. 3).

Bile canalicular length was measured using immunofluorescence to quantitatively investigate bile canalicular formation. There were no significant differences in the bile canalicular length (μm/cell) between fresh and flask-delivered PXB-cells under the same oxygen supply condition (Fig. 4B). However, under sufficient oxygen supply conditions in both fresh- and flask-delivered PXB-cells, the length was significantly increased by approximately 1.1-fold and 1.4-fold on days seven and 14, respectively, when compared with those in oxygen supply (−) conditions. These results emphasize the acceleration of bile canaliculus formation under adequate oxygen supply via oxygen-permeable plates.

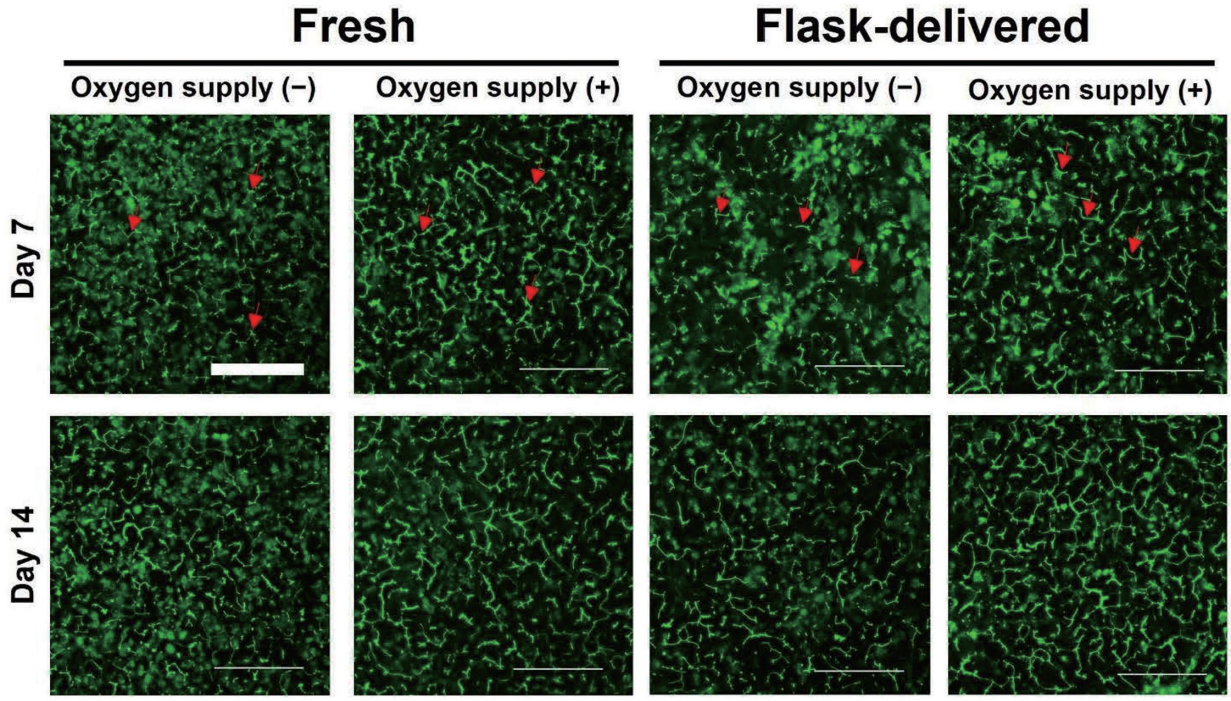

Fig. 3. Excretion of fluorescein-labeled bile acids (cholyl-lysyl-fluorescein [CLF]) into bile canaliculi. The red arrows show the bile canaliculi structure. Scale bar = 200 μm.

DISCUSSION

Functional bile canaliculus formation in cultured human hepatocytes is crucial for studying the hepatobiliary disposition and drug-induced cholestasis *in vitro*. PXB-cells are a promising cell source for these studies and are currently available in conventional tissue culture multi-well plates (Fresh PXB-cells) or in tissue culture flasks (Flask-delivered PXB-cells). However, the effect of reseeding cells from tissue culture flasks, particularly on bile canaliculi, has not yet been validated. Additionally, the effect of adequate oxygen supply on bile canaliculus formation in PXB-cells is not fully understood. In this study, we demonstrated that flask-delivered PXB-cells, after seven days of culture, restored major hepatic functions and bile canaliculi formation to levels comparable with those of fresh PXB-cells. Notably, direct oxygen supply to PXB-cells through an oxygen-permeable membrane enhanced bile canaliculus formation in both fresh and flask-delivered PXB-cells. These findings have significant implications for the appropriate utilization of PXB-cells in drug testing and toxicological research.

Our results demonstrated the enhancement of the extended functional bile canaliculi formation of PXB-cells under adequate oxygen supply via an oxygen-permeable membrane (Figs. 3 and 4). In conventional biliary analysis, functional bile canaliculi are essential because the recovery of biliary compounds depends on the size of bile pockets (Kanda *et al*., 2018). However, stable bile canaliculi formation is difficult in cryopreserved PHHs because of cellular damage caused by freeze-thaw cycles, leading to inaccurate predictions of biliary clearance (Kitaguchi *et al*., 2023; Sakai *et al*., 2023). Even in fresh PHHs, the estimated human biliary clearance with sandwich-cultured PHHs was 20 times lower than the actual human biliary clearance, possibly because of poor bile canaliculus formation (Abe *et al*., 2009). Additionally, studies have reported that small bile pockets in human hepatocytes lead to difficulty in accurate measurements, especially for compounds with low biliary clearance (Kitaguchi *et al*., 2023; Matsui *et al*., 2012). Hence, oxygen-mediated enhancement of bile pockets should increase the recovery of biliary compounds from bile canaliculi, enabling the accurate measurement of biliary

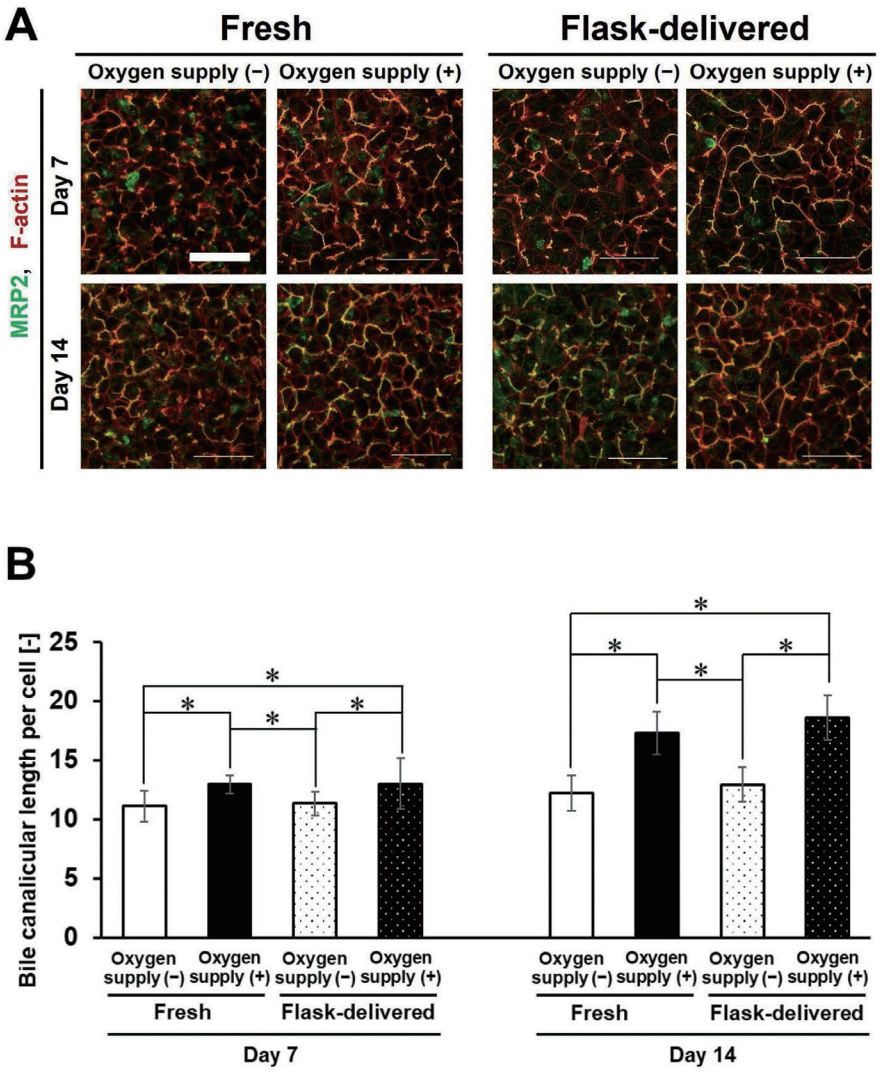

Fig. 4. Analysis of bile canaliculi formation. **A.** Immunostaining of F-actin (red) and MRP2 transporter (green) on days seven and 14. The co-localization parts (yellow) of F-actin and MRP2 represent the bile canaliculi. Scale bar = 100 μm. **B.** Bile canalicular length per cell. The measurement was performed with twelve images of immunofluorescence staining from two independent experiments, mean ± standard deviation. *: $P < 0.05$

clearance.

As to the mechanism of the bile canaliculi elongation by sufficient oxygen supply, studies demonstrated that the mitochondria-mediated oxidative phosphorylation accelerated the repolarization of cultured hepatocytes (Fu *et al*., 2013). In a report, direct oxygen supply increased the levels of mitochondrial metabolism in rat hepatocytes (Scheidecker *et al*., 2020), and the oxygen demand in human hepatocytes was lower than that in rat hepatocytes (Stéphenne *et al*., 2007; Stevens, 1965). Oxidative phosphorylation in PXB-cells should also be increased under oxygen supply (+) conditions. Therefore, enhanced mitochondrial activity under sufficient oxygen supply may contribute to bile canaliculi elongation.

Albumin production in PXB-cells was enhanced by direct oxygen supply through oxygen-permeable plates (Fig. 2). Our results agree with studies on cryopreserved PHHs, in which they increased albumin production by incubating cells in a 95% oxygen atmosphere (Kidambi *et al*., 2009). However, with this method, it is difficult to control the oxygen concentration in the vicinity of the cells, and a high concentration of oxygen might

lead to excess oxidative stress (Danoy *et al*., 2022). By contrast, direct oxygen supply through oxygen-permeable membranes can easily meet the oxygen demand of cells at low physiological oxygen concentrations (Nishikawa *et al*., 2022; Nishikawa *et al*., 2008).

In recent years, there has been much development in physiological *in vitro* tissue culture systems using unique culture plates and devices that mimic *in vivo* microenvironments to replace animals and overcome species differences and animal welfare concerns (Shinha *et al*., 2021; Wadman, 2023). The use of functional cell sources is important to reproduce physiological responses in these culture environments. PXB-cells, which are available in tissue culture flasks, are a promising cell source to meet these demands. In this study, we found that flask-delivered PXB-cells increased their hepatic function concerning albumin production during culture and reached comparable levels to freshly isolated PXB-cells by approximately one week of culture, consistently demonstrating functional bile canaliculi formation. The findings of this study offer valuable insights into the potential use of PXB-cells for *in vitro* evaluations using *in vivo*-mimicking cell culture devices for drug discovery and toxicological research.

This study focused on comparing bile canaliculi formation between fresh and flask-delivered PXB-cells without validating drug metabolism and toxicity evaluations. Further studies should assess the drug metabolism and toxicity of various compounds to investigate the detailed effects of reseeding on PXB-cells. Furthermore, considering the better enhancement of albumin production with sufficient oxygen supply in fresh PXB-cells compared with that in flask-delivered PXB-cells (Fig. 2A), alterations in mitochondrial function, which are responsible for aerobic respiration with oxygen, may have occurred during the reseeding process of PXB-cells. The effect of this process on mitochondrial function need to be explored in future studies. Additionally, as studies have demonstrated the alteration of cellular metabolism by passaging cells using trypsin (Goldstein *et al*., 1982; Huang *et al*., 2010), the difference in energy metabolism between fresh and flask-delivered PXB-cells should also be validated for a comprehensive understanding of the effect of the reseeding process of PXB-cells.

In conclusion, flask-delivered PXB-cells demonstrated functional activity and bile canaliculi formation during culture, which was comparable with that of fresh PXB-cells. More importantly, the formation of bile canaliculi was enhanced by sufficient oxygen supply. Functional bile canaliculi with direct oxygen supply would enable the accurate evaluation of biliary compounds. These findings will contribute to the acceleration of *in vitro* human liver studies for drug development and toxicological research.

ACKNOWLEDGMENTS

This study was partly supported by the Japanese Agency for Medical Research and Development for the "Program for the Foundational Technology Development for the Promotion of Regenerative Medicine Industrialization" (Grant No.22be1004201h0001) and the Japan Society for the Promotion of Science (Grant No.23KJ0541). We would like to thank Editage (www.editage.jp) for English language editing.

Conflict of interest---- This work was performed in collaboration with PhoenixBio Co., Ltd., which provides PXB-cells® to researchers as its proprietary product.

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