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### A new experimental model to study human drug responses

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### Abstract

Accurate prediction of pharmacokinetic (PK) and pharmacodynamic (PD) characteristics is critical for drug development. Oral drugs are particularly difficult because they are absorbed by the intestine and metabolized in the liver before systemic metabolism in vivo; this is called the first-pass effect and is a critical factor for predicting oral bioavailability (BA). Here, we fabricated a new networking and circulating cell culture system (NCCS), mimicking the circulatory system and interaction of organs for studying the pharmacokinetic and pharmacodynamics of oral drugs in vitro. NCCS consisted of a micro-pump for circulating fluids, two types of multi-insert culture dishes for culturing different cell types, and an orbital shaker for mixing; flow rate and shaking-speed were controlled by weight-sensors and drivers. A first-pass effect test was performed using functionally differentiated HepaRG and Caco-2 cell lines, using a new modified spheroid forming unit (SFU) protocol. To verify the similarity of PK (first-pass effect) data of NCCS with the data from the human body, 15 reference drugs were chosen and their associated data were obtained by liquid chromatography-mass spectrometry analysis. NCCS generated absorption and metabolism data showed >70% similarity to human data respectively. NCCS can also be used to demonstrate species differences. Animal models are the primary basis for drug discovery, development, and testing. However, the weak correlation between humans and animals, particularly regarding absorption and metabolism, is a substantial limitation for the use of animal models. Here we compare human and mouse acetaminophen (APAP) metabolism using NCCS, and its application can be extended to assess cellular responses, such as efficacy and toxicity, simultaneously.

### 1. Introduction

Accurate prediction of pharmacokinetic (PK) and pharmacodynamics (PD) characteristics is critical for drug development. Researchers are accustomed to using classical two-dimensional cell cultures to validate drugs or compounds, followed by *in vivo* animal studies. Animal experiments are usually conducted, and considered reliable, to establish *in vivo* the PK and PD characteristics of a drug candidate. However, such experiments have several limitations, including high cost, ethical issues, and species differences [1]. Thus, most compounds that demonstrate a prospective profile in preclinical studies fail to advance into clinical phases, and success rates of reaching the market are only 10%–20% [2]. In particular, there is a weak correlation between animals and humans regarding absorption and metabolism, which constitute critical factors for the failure of drug discovery [3].

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In recent years, however, in recent years, a call for the more advanced model has been raised, since conventional methods often fail to adequately reproduce the complexity of a human body [4]. To overcome this issue, previous studies have proposed various models representing human cells [5, 6] and the human body, which enhance the functions of cells and mimic *in vivo* organ networks, using microfluidic techniques [7–11].

After oral administration, drugs are absorbed by the intestine and metabolized in the liver before systemic metabolism in vivo; this is called the firstpass effect and is a critical factor for predicting oral bioavailability (BA). Several studies have developed Caco-2 and HepaRG cell lines as the absorption and metabolism models to estimate the first-pass effect of orally administered drugs in vitro [12–14]. However, stochastic variations from bench-to-bench still pose hurdles towards achieving precise results. HepaRG differentiates into two different cell types; directing the differentiation toward the hepatocytelike cell type, which expresses cytochrome p450 enzymes, instead of toward the bile-duct-like cells, was critical to establish a metabolic model. It has been reported that fluid shear stress promotes hepatocyte differentiation and enhances function [15]. Additionally, we have shown that rotational culture promotes hepatocyte-like characteristics, such as albumin secretion and CYP3A4 expression previously [16]. Thus, using HepaRG cultures for testing the first-pass effect will need to take into consideration differentiation towards hepatocyte like cells.

The first-pass effect involves the networking of intestinal and liver functions in absorption and metabolism. Thus, a platform that recapitulates the networking of organs is required for an accurate drug test. Recently, Kimura et al reviewed the use of organ-on-a-chip, a microfluidics-based technology, and proposed it as a useful tool in drug discovery. However, there are no concrete examples in which organ-on-a-chip technologies have been fully utilized in the actual drug discovery process [17]. Here, we attempted to develop a more advanced platform for absorption and metabolism assessment. First, we fabricated a new 60 mm dish-scale cell culture workstation called networking cell culture system (NCCS) and explored its applications in PK and PD. Next, we suggested effective protocol to promote differentiation of highly functional hepatocyte-like cells from HepaRG without using DMSO in a shorter span of time using spheroid forming unit (SFU), a new platform, and to increase tight junction permeability of Caco-2 cells using SFU. In addition, we have elucidated the mechanism underlying SFU promoting differentiation of HepaRG cells lines to hepatocytes. Subsequently, the NCCS platform has been validated

in assessment of PK/PD and shown the possibilities of extending the newly developed platform to assess the species difference of drug liver metabolism and drug induced toxicity.

### 2. Methods

#### 2.1. Compartments of NCCS

NCCS consisted of networked four multi-insert dishes (SPL, Korea), a piezoelectric pump (Standard type, Takasago, Japan), weight load cell sensor (ZEL6J1, Cozy, Korea), with the entire size measuring 21 cm  $\times$  21 cm  $\times$  23 cm. A motor (BLDC, Hyoseong, Korea) was mounted to the main body to produce an orbital shaking movement (supplementary figure 1 (available online at stacks.iop.org/BF/12/045029/mmedia)). These parts were assembled by EnZ Tech., Korea based on the author's idea.

# 2.2. Cell viability test (crystal violet staining, cell viability assay, proliferation assay and apoptosis assay)

Cells were seeded into a multi-insert culture dish and then cells were cultured at the indicated culture conditions for 7 d in NCCS. The cells were then washed with phosphate buffered saline (PBS), fixed with 10% formaldehyde and stained with crystal violet (0.5%, w/v). Cell viability was analyzed using a LIVE/DEAD® viability/cytotoxicity kit (Molecular Probes<sup>™</sup>, Eugene, OR, USA) according to the manufacturer's instructions. Calcein-AM (2 µM) and ethidium homodimer-1 (EthD-1)(4  $\mu$ M) were added and incubated for 15 min at 37 °C with 5% CO<sub>2</sub>. The conversion of non-fluorescent calcein-AM into fluorescent calcein and the binding of EthD-1 to DNA was monitored using a fluorescence microscope (Olympus, Tokyo, Japan). Calcein-AM and EthD-1 were excited using a green fluorescence filter and a red fluorescence filter, respectively. The survival of attached cells was analyzed by crystal violet standing (0.5%).

For the proliferation/viability assay, we used EZ-CYTOX reagent (Daeil Lab service, Seoul, Korea) according to the manufacturer's instructions. The assayed cells were incubated in a  $CO_2$  incubator at 37 °C for 1 h and then measured at 450 nm using a microplate reader (Molecular devices, San Jose, CA, USA).

For the apoptosis assay, after drug treatment, cells were harvested and incubated with 100  $\mu$ l of Muse Annexin V & Dead Cell reagent (Millipore, Bedford, MA, USA) for 20 min at room temperature. Apoptosis was determined by Muse Cell Analyzer (Millipore, Bedford, MA, USA), and the statistics were shown as percentages of the cells alive, apoptotic, or dead.

### 2.3. Detection of glucose and pH

Cells were cultured in NCCS or static conditions, and medium was collected from the culture dishes. The glucose concentration was determined by an Accu-Chek glucose monitoring system (SmartView, ACCU-CHEK, USA). Ten microliters of medium was loaded into a pocket-sized pH meter (#S2K712; ISFETCOM, Japan) and measured every 2–3 d.

### **2.4. Cell culture and differentiation protocols** *2.4.1. Cell lines*

Immortalized hepatic cell line HepaRG were purchased from Biopredic International (HPR 101, Rennes, France) and the human colon cancer cell line Caco-2 and kidney proximal tubule cells HK-2 were obtained from the American Type Culture Collection (Rockville, MD, USA). The human lung fibroblast cell line MRC5 were obtained from the Korean Collection for Type Culture. Mouse small intestine epithelial cells (SIEP) were purchased from Applied Biological Materials Inc. (Richmond, BC, Canada). The human monocyte U937 cells were obtained from the Korean Collection for Type Culture. The alpha mouse liver 12 cell line (AML12) was obtained from the American Type Culture Collection (Rockville, MD, USA). For maintenance and standard or static cell culture conditions (as noted), the cell lines were cultured with appropriate medium per supplier's instructions and routinely tested to confirm the absence of mycoplasma. All experiments were performed with cell lines at 60%-80% confluence.

### 2.4.2. Differentiation protocols

Each cell line was used under both standard culture conditions and under differentiation conditions including SFU platform as noted. The differentiated state was defined differently depending upon the cell line.

SFU platform: A rotatory cell culture platform consisting of a tube and filter cap (SPL, Pocheon, Korea) for the generation of spheroid previously [16], but it was used for the suspension cell culture in this study (supplementary figure 3).

HepaRG cells: For HepaRG cells, two different differentiation protocols were compared. For standard differentiation, HepaRG cells were cultured in HepaRG maintenance medium for 7 d, and 2% of DMSO was added to the medium for an additional 23 d. For SFU differentiation, a suspension of HepaRG cells ( $2 \times 10^6$  cells) were transferred to SFU platform and then cultures for 48 h. Cells were seeded into mesh-sided multi-insert dish and cultured in HepaRG maintenance medium for additional 13 d without DMSO.

<u>Caco-2 cells</u>: For standard differentiation, Caco-2 cells  $(1 \times 10^6 \text{ cells})$  were seeded into mesh-bottom multi insert dish and cultured to differentiate for 14–21 d with regular medium changes three times weekly. For SFU differentiation, suspension of Caco-2 cells

 $(1 \times 10^6$  cells) was transferred to SFU platform and then cultures for 48 h. Cells were seeded into meshbottom multi insert dish and the culture medium changed every 2 d and the cells were cultured for 14 d.

<u>U937 cells</u>: U937 cells were differentiated into active macrophages by 100 ng ml<sup>-1</sup> of prhorbol-12myrostate-13-acetate (PMA, Sigma-Aldrich, USA) for 3 d. PMA-induced U937 cells were analyzed by anti-CD11b antibody using a FACS Calibur and Cell Quest FACS system (BD Biosciences, San Jose, CA, USA).

<u>AML-12 and SIEP cell line</u>: The differentiation protocols are described in supplementary materials and methods file.

Docking multi-insert dish to NCCS: At the final day of differentiation, the prepared cells were seeded into the appropriate insert type of a multi-insert dish and transferred into the NCCS system for drug assessments (supplementary figure 3).

#### 2.5. Immunofluorescence staining

Cells were cultured on a 4-well culture slide (Corning, USA) for 14 d, then washed with PBS (pH 7.4,  $37 \degree$ C), fixed with 4% paraformaldehyde for 15 min at 37 °C, and permeabilized with 0.1% Triton X-100 (v/v) for 15 min at 4 °C. After fixation, the cells were washed twice with PBS and incubated for 1 h at room temperature with 0.2% fish skin gelatin solution. Anti-CK-19 (Abcam, Cambridge, UK), anti-albumin (R&D systems, Minneapolis, MN, USA), anti-CDX2 (Abcam, Cambridge, UK), anti-ZO-1 (Invitrogen, Carlsbad, CA, USA), anti-PEPT1 (Santa Cruz, Santa Curz, CA, USA), anti-MDR1 (Millipore, Carlsbad, CA, USA), and anti-KRT20 (Abcam, Cambridge, UK) primary antibodies were added and incubated overnight at 4 °C. After incubation, cells were washed three times with PBS for 10 min, followed by incubation with goat-anti-mouse-Alexa488 or goat-anti-rabbit-Alexa488 secondary antibodies (Molecular Probes, Eugene, OR, USA). After 1 h incubation at room temperature, the cells were washed three times with PBS for 5 min and imaged. For the apical-basolateral polarity sectioning, Caco-2 cells were seeded in the appropriate multi-insert dish and cultured for 14 d. Cells were washed with PBS, and the insert filter cut out with a blade. Samples were fixed in 4% paraformaldehyde, frozen, and embedded in OCT compound. Vertical cryosections of 5  $\mu$ m in thickness were prepared using a Leica cryomicrotome CM3050. Sections were washed with PBS and blocking agent (0.2% fish skin gelatin). Immunofluorescence staining was conducted as described above.

# 2.6. Hepatocyte functional assays (albumin and CYP3A4 activity assay)

All of differentiated HepaRG cells were starved with serum free culture medium for 24 h, and supernatant was collected for albumin secretion assessment using human albumin ELISA quantitation kit (Bethyl Laboratories, Montgomery, TX, USA). The assay was performed according to the manufacturer's instructions. CYP3A4 activity was detected using the P450-Glo CYP3A4 assay with Luciferin-IPA (Promega, Fitchburg, WS, USA), according to the manufacturer's instructions. Briefly, CYP specific probe substrate (Luciferin-IPA) was diluted in serum free medium and incubated with the cells for 1.5 h. The supernatant was collected. Luciferin detection reagent was added for an additional 20 min, and then measured using a luminometer (Molecular device, USA).

#### 2.7. Cell cycle analysis

HepaRG cells were harvested by centrifugation at 1200 rpm for 5 min after SFU culturing for 24 h. The cell pellets were washed with cold PBS, fixed with 70% ethanol, and collected by centrifugation. The pellets were resuspended in PBS. The cell samples were then stained with 100  $\mu$ g ml<sup>-1</sup> propidium iodide (PI; BD Biosciences) in PBS containing 10  $\mu$ g ml<sup>-1</sup> RNase A at 4 °C for 30 min in the dark. Stained samples were analyzed using a FACS Calibur and Cell Quest FACS system (BD Biosciences). The percentage of cells in each cell cycle phase was determined. All samples were measured in triplicate.

# 2.8. Trans-epithelial electrical resistance measurements and permeability of Caco-2 monolayers

For the measurement of transepithelial electrical resistance (TEER), Caco-2 cells ( $5 \times 10^5$  cells ml<sup>-1</sup>) were seeded onto transwell permeable supports and cultured for 14 d. TEER measurements were done using a MERSSTX01 Millipore Millicell ERS probe and ERS-2 Epithelial volt-ohm meter. FITC-labeled dextran (3 kDa, Life Technology, Carlsbad, CA, USA) was added to the apical compartment at a final concentration of 0.1 mg ml<sup>-1</sup>. After 30, 60, 120, and 180 min, 100  $\mu$ l of medium was collected from both the apical and basal compartments and replaced with the same amount of medium. Samples were collected into a 96well micro-titer plate.

### 2.9. Measurement of glutathione (GSH) level

SFU-differentiated HepaRG or AML12 cells were treated with or without 2 mM APAP for 3 h at 37 °C, and the glutathione level in the culture medium was determined using the total Glutathione Quantification kit (Dojindo Laboratories, Kumamoto, Japan), according to the manufacturer's instructions.

### 2.10. Operation of NCCS and analysis of samples

Prior to assembly, two or four dishes and connection tubes were sterilized with 75% ethanol and washed with PBS on a clean bench under ultraviolet irradiation. Twenty five ml of conducting medium (William's media with 2% FBS) was added into each connected outer dish and then put into the multi-insert dish that contained the prepared cells at appropriate portion in NCCS. The NCCS device was set at 2 ml min<sup>-1</sup> flow rate and 0.8 RPS (revolution per second) shaking speed, for 10 min. Each chemical was added to the NCCS device into the first dish, which was set up as the intestine unit, at an appropriate concentration. Media samples were collected from the connected tubing after the liver unit (the last unit of the series) at each time point. Media samples were analyzed by LC-MS/MS, and used cells were analyzed using various molecular techniques as noted. The relative concentration (%) of the test drug was calculated by setting the drug's pretest concentration to 100%. Cmax represented the maximum concentration of tested drugs, and tmax represented the time needed to reach Cmax. Clearance ( $\Delta C$ ) was the change from Cmax to the concentration at 48 h  $(C_{48h}).$ 

# 2.11. Measurement of APAP and its metabolites in cultured media by LC-MS/MS

The samples were prepared and analyzed for APAP, 4-aminophenol, and APAP-GSH content using liquid chromatography tandem mass spectrometry (LC-MS/MS). Briefly, samples were extracted with 100% acetonitrile containing carbamazepine as an internal standard (IS), and chromatography was done using an Atlantis dC18 column (50  $\times$  2.1 mm i.d., 3  $\mu$ m, Waters, Milford, MA) with a SecurityGuardTM C18 guard column (2.0  $\times$  4.0 mm i.d., Phenomenex, Torrance, CA), maintained at 30 °C. HPLC mobile phases consisted of A (deionized water containing 0.1% [v/v] formic acid) and B (acetonitrile containing 0.1% [v/v] formic acid). A linear gradient of the two solvents was used: starting at 100% A, held for 1.0 min, ramped up to 50% A for 1.1 min and held until 4 min. The flow rate was set at 0.4 ml min<sup>-1</sup> throughout the gradient. The retention times of APAP, 4-aminophenol, APAP-GSH, and IS were 3.02, 2.94, 2.97, and 3.70 min, respectively. The ESI source was operated at 5500 V and 600 oC. The samples were analyzed via multiple reactions monitoring mode (MRM). The monitoring ions were set as m  $z^{-1}$  152 > 110 for APAP, m  $z^{-1}$  271 > 182 for 4aminophenol, m  $z^{-1}$  110 > 65 for APAP-GSH, and m  $z^{-1}$  237 > 194 for IS. The scan dwell time was set at 0.1 sec for every channel. Acquisition and analysis of data were performed with AnalystTM software (version 1.5.2, Applied Biosystems, Foster City, CA).

# 2.12. Gamma-glutamyltransferase (gamma-GT) activity

GGT activity was measured by following the release of para-nitroanilide from gamma glutamyl-pnitroanilide using a gamma-GT activity colorimetric assay kit (BioVision, Milpitas, CA, USA). Cell and kidney spheroids were homogenized in 200  $\mu$ l of ice-cold gamma-GT assay buffer, and 10  $\mu$ l aliquots were combined with 90  $\mu$ l of gamma-GT substrate

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solution and added to the assay plate for a 5 h incubation. Absorbance changes at 418 nm were measured every 30 min at 37 °C.

### 2.13. Prostaglandin E2 (PGE2) detection

During differentiation in NCCS or under static conditions, samples from U937 cultures were collected from culture dishes. Production of PGE2 in the samples was quantified by ELISA kits (Abcam, Cambridge, UK) according to the manufacturer's protocol.

### 2.14. Statistical analysis

All data show representative data of more than three independent replicates. Data were represented as the mean  $\pm$  S.D. ( $n \leq 5$ ) or mean  $\pm$  S.E.M. ( $n \geq 10$ ). Student's *t*-test was used to compare two groups. A *p*-value < 0.05 was considered to be statistically significant. The correlation (R) of absorption and metabolism was calculated using Prism version 7.0 (GraphPad software) by a best-fit sigmoid function.

#### 2.15. Supplementary materials and methods

Antibodies, reagents, western blot analysis, qRT-PCR/RT-PCR, and immunofluorescence staining are described in supplementary materials and methods. Primers for qRT-PCR and RT-PCR are described in supplementary tables S1 and S2. The analysis condition for LC-MS/MS is described in supplementary table S5.

### 3. Results

# 3.1. Fabrication of the NCCS platform mimicking blood circulation and organ networking of the human body to study PK/PD

Orally administered drugs show efficacy in vivo only after exposure via networked organs, i.e. intestine, liver, and kidney. Blood circulation is a mediator for drug transfer and kinetics (PK) or dynamics (PD) of systemic drug concentration. NCCS was designed to mimic the circulatory system, as well as the interaction of organs for measuring the PK/PD profile of orally administered drugs. To realize this in vitro, we designed the system to mimic the circulatory system representing the ADME (Absorption, Distribution, Metabolism, and Excretion) parameters of PK. The platform consisted of four connected the outer dishes (60 mm each) representing the organs involved in the ADME process (figure 1(a)), though these could be mixed and matched as needed. The outer dish also that consisted of an air-filtered cap and body and possessed two nozzles to connect adjacent dishes. Filter caps were used to supply oxygen, and the capacity of each dish was 25 ml (figure 1(b)). The outer dish can contain a multi-insert culture dish, which holds five inserts (13 mm diameter each) with 0.4 µm pore size mesh bottoms for drug absorption or 20 µm pore

size mesh sides for drug metabolism and other drug assessments (figure 1(b)). For example, Caco-2 and HepaRG cells have been able to be prepared by mesh bottom-dish for drug absorption and by mesh sidedish for metabolism respectively.

Each dish was attached to a weight sensor and a pump. The weight sensor was equipped to detect the weight of the culture medium in the connected dish and maintain equal amounts of culture medium in each dish. Specifically, if a problem occurs in the flow of culture medium and a deviation in weight is detected, the pump driver will control the flow rate or stop it as needed. The pumps and dish were connected through silicon tubing for the circulation, and the platform was manufactured to include an orbital shaker (0.1 to 10 RPS) for the sufficient mixing between drugs and cells. A constant flow rate of medium (1 to 20 ml min<sup>-1</sup>) was maintained for each dish with precise control (figure 1(c)). The real pictures of each parts were presented in supplementary figure 1.

Next, we optimized culture conditions using NCCS, testing at different flow rates and shaking intensities; a flow rate of 2 ml min<sup>-1</sup>, with a shaking speed of 0.8 RPS, was selected as the optimal condition for cell viability (live/dead staining) and attachment (Crystal violet staining; figure 1(d)). Since HepaRG cells are particularly finnicky as to culture conditions, we tested the effect of changes in cellular conditions on cell viability and glucose consumption (figures 1(e) and (f)) of HepaRG cells with longterm monitoring. We observed a more stable proliferation of cells under optimized NCCS conditions (2 ml min<sup>-1</sup> and 0.8 RPS) than under static culture conditions, which could be due to shear stressinduced by the volume, shaking, and flow of medium enhancing the cell growth. To quantitatively investigate the effect of shaking on the cells, we measured the velocity field in the shaking dish. Supplementary figure 2 shows the temporal evolution of the velocity field in the shaking dish versus optimal operating conditions for stable cell culture. Arrows denote the direction of flow, and their length represents the magnitude of velocity. It was observed that the velocity magnitude varied from 0 to 10 mm s<sup>-1</sup> (supplementary figure 2(a), left), and the velocity field was repeated at a period of approximately 216 ms (supplementary figure 2(a), right). This range is almost 10 times higher than that of the static dish (supplementary figure 2(b)). Because shear stress  $\tau$  scales as U  $h^{-1}$ , where U is the velocity at height h from the cell, we deduced that shear stress on the cells in the shaking dish at optimal operating conditions is 10 times higher than that in the static dish Thus, the NCCS platform actually optimizes cell growth.

Collectively, the NCCS platform developed here could ideally be operated with four channels, mimicking the ADME process. As we demonstrate below,



**Figure 1.** Networking cen culture system (NCCS) for the study of PK/PD. (a) flustration of the concept of *m vivo* ADME and *m vitro* ADME. (b) Illustration of the outer dishes and multi-insert culture dishes. The multi-insert culture dish contained five 24-well inserts which is 13 mm diameter with 0.4 µm pore size meshed-bottoms for drug absorption and 20 µm pore size meshed-sides for drug metabolism and other drug assessments. The 60 mm outer dish which can contain multi-insert-dish is composed of an air mesh cap and the body, with a nozzle for connection unit. (c) Right-side, Front-side, and Top view of the NCCS device. (d) Optimization of NCCS-conducting condition for cell survival. Live cells are shown in green and dead cells shown in red. The healthy attached cells were stained by crystal violet (0.5%). Flow rate 2 ml min<sup>-1</sup> and shaking 0.8 RPS, were the optimal conditions for cell survival. Cell viability (e), Changes of glucose-concentration (f) at various time points of culture in static culture or NCCS conditions (2 ml min<sup>-1</sup>, 0.8 RPS).

this versatility should make NCCS a useful drug validation platform. In addition, it could also be used for long term cell culture.

# 3.2. Preparation of functional liver cells using spheroid forming unit (SFU) protocol for the metabolism aspect of NCCS

We developed a new protocol to differentiate HepaRG cells into hepatocyte-like cells, without DMSO, in only 15 d using SFU. Previously, we had reported that rotational culture promotes hepatocyte-like characteristics, such as albumin secretion and CYP3A4 expression [16]. Briefly, the suspension of HepaRG cells were transferred to SFU using HepaRG growth medium, and rotationally cultured for 48 h. The cultured cells were maintained in the HepaRG growth medium without DMSO for an additional 13 d in the multi-insert dish (figure 2(a) and supplementary figure 3).

We compared the hepatocytes generated using our new protocol with cells generated using the standard differentiation protocol with DMSO (figure 2(a), top). The HepaRG cells changed from spindle-like shape to a shortened and rounded morphology with a lipid droplet at 7 d after SFU culture, which was similar to that in case of differentiated cells in DMSO-enriched medium, and hepatocyte-like cells were observed at 15 d (figure 2(a), bottom). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) results showed a significant increase in the expression levels of hepatocyte-related markers;  $HNF1\alpha$ ,  $HNF4\alpha$ , Albumin, ALDOB, and GSTA1 in a culture time-dependent manner through SFU protocol as compared to the standard protocol (figure 2(b)). Moreover, the expression of bile canaliculi markers, such as  $HNF1\beta$ , Sox9, CK7, CK19 and GGT1, was dramatically reduced after differentiation with our new protocol, except for *Sox9* (figure 2(c)).

We also analyzed the functionality of the hepatocytes generated using our new protocol. Transcripts of five cytochrome P450 genes (*CYP1A2*, 2C9, 2D6, 2E1, and 3A4) and four transporter genes (*MDR1*, ABCC2, *SLC22A1*, and A7) were analyzed by qRT-PCR in HepaRG cells at different times after SFU or standard



**Figure 2.** Effective hepatic differentiation of HepaRG for the metabolism part of NCCS. (a) Schematic diagram of the hepatic differentiation protocol (top) and morphology of HepaRG cells during *in vitro* culture (bottom). Standard differentiation protocol (ST) is a well-established protocol using 2% of DMSO for 30 d as a differentiation control, and the SFU differentiation protocol (SFU) was a combination of rotational culture and conventional culture without the use of DMSO for 15 d. CTL, undifferentiated cells. (b) Quantitative RT-PCR analysis of related hepatic markers in HepaRG cells at 7 d or 15 d of SFU and at 15 d or 30 d of ST compared to that of control HepaRG cells (CTL). (c) Quantitative RT-PCR analysis of bile canaliculi markers in HepaRG cells (TL). (d) Relative mRNA expression levels of five cytochrome P450 genes (*CYP1A2, 2C9, 2D6, 2E1,* and *3A4*) and four transporter genes (*MDR1, ABCC2, SLC22A1*, and *SLC22A7*) in differentiated HepaRG cells at 7 d or 15 d of SFU and at 15 d or 30 d of ST compared to that of control HepaRG cells (CTL). (e) Representative images of immunostaining of differentiated HepaRG cells at 15 d or SFU or 30 d of ST or albumin and cytokeratin 19 (CK19) and undifferentiated cells (CTL). (f), (g) Albumin secretion amounts (f) and CYP3A4 enzyme activity (g) of hepatic-like cells, differentiated by SFU protocol at 7 d or 15 d compared to that of undifferentiated HepaRG cells at CTL) and differentiated cells cultured using standard differentiation protocol (ST). (h) Protein expression of mature hepatocyte makers was analyzed by western blotting using HepaRG cells after SFU culture at indicated time points. (b)–(d) Data are means  $\pm$  SEM (n = 10). \* p < 0.05. (f), (g) Data are means  $\pm$  SD (n = 5), \* p < 0.05.

differentiation. The mRNA expression of *CYP1A2*, *CYP2C9*, *CYP2E1*, *CYP3A4*, *MDR1*, *SLC22A1* and *SLC22A7*, was found to be increased at 7 d of SFU differentiation, wherein the mRNA expression of all the nine genes significantly increased at 15 d of SFU differentiation, similar to that in cells after 30 d of standard differentiation (figure 2(d)). Immunostaining further confirmed the increased expression of bile canaliculi marker CK19 in HepaRG cells after SFU or standard differentiation (figure 2(e)).

Albumin secretion and CYP3A4 activity were also significantly increased in the cells at 7 d and 15 d of SFU differentiation, similar to that in cells undergoing standard differentiation (figures 2(f) and (g)). Protein expression of mature hepatocyte markers, HNF4 $\alpha$  and CYP3A4, was analyzed and found to be significantly increased in HepaRG cells differentiated with SFU (figure 2(h)). These results suggested that our new protocol could be used to effectively differentiate HepaRG cells into functional hepatocytes without the use of DMSO.

### 3.3. Induction of hepatocyte-related gene expression through activation of kinase signaling pathways by the SFU condition

Subsequently, we elucidated the mechanism underlying the hepatic differentiation of HepaRG cells in the SFU culture conditions. To this end, mRNA expression of hepatocyte-related or bile canaliculi-related genes was quantitated by qRT-PCR after incubation for 0, 24, and 48 h under SFU culture conditions. The results showed an increased mRNA expression of hepatocyte-related genes in HepaRG cells (figure 3(a)), whereas the expression of biliary genes was rapidly decreased under the SFU culture conditions (figure 3(b)). We analyzed various hepatocyte-specific genes by RT-PCR, using specific primers (supplementary table S1), including the liverenriched transactivating factors FOXA2 (HNF- $3\beta$ ), C/EBP- $\alpha$ , C/EBP- $\beta$ , USF2, HNF4 $\alpha$ , and CREBH at various time points during SFU culture, and the results were quantified using densitometry software (supplementary figure 4). We observed a maximum increase in FOXA2 mRNA levels as compared to that if other genes at early time point after SFU culture.

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FOXA2 is a transcription factor that can bind to the promoter region of the target gene and regulate the mRNA level. In addition,  $C/EBP-\alpha$ ,  $C/EBP-\beta$ , USF2,  $HNF4\alpha$ , and CREBH target genes were transcriptionally regulated by *FOXA2*. The expression of *albumin* and *CYP3A4* mRNA increased in HepaRG cells in a time-dependent manner under the SFU culture (figure 3(c)).

To confirm whether shear-stress from the SFU culture conditions induced endoplasmic reticulum (ER)-stress signal in HepaRG cells, the expression levels of *ATF4*, and one of the target genes of *ATF4*, namely *CHOP*, were confirmed in HepaRG cells but not *ATF6* and *XBP-1*. The expression of *ATF4* mRNA was the highest at 3 h after the start of SFU incubation, while that of *CHOP* mRNA increased after 24 h. These results confirmed that SFU culture conditions induce ER-stress signaling; however, it is not an upstream signal pathway that can increase the expression of *FOXA2* mRNA (figure 3(c)). The protein levels of hepatocyte-related or ER-stress-related genes in HepaRG cells, under SFU culture conditions, were also analyzed by western blotting (figure 3(d)).

To further define the signaling events after SFU culture, we confirmed the cellular phenotypic changes, such as the cell cycle and lipid accumulation. HepaRG cells were assessed for cell cycle distribution using flow cytometry, with propidium iodide staining, at 0 and 24 h after SFU culturing. Approximately 80.8% of the cells were in the G1 phase, 4.74% in the S phase, and 14.4% in G2/M phase compared to the conventional cultured cells (CTL) that consisted of 67.8% in G1 phase, 9.6% in S phase, and 22.5% in G2/M phase. Thus, SFU culture-induced cell cycle arrest at the G1 checkpoint (figures 3(e) and (f)). The expression of cyclin mRNAs and proteins was decreased under SFU culture conditions (figures 3(g) and (h)). Differential gene expression was analyzed after final differentiation, and genes related to cell cycle were downregulated while those related to lipid metabolism and xenobiotics were upregulated (supplementary figures 5(a)-(c)).

Under SFU culture conditions, the expression of *FOXA2* mRNA increased the activity of various kinases in the integrin signal, and the involvement of p38 MAPK signal pathways in cell differentiation was confirmed in HepaRG cells. The kinases that were activated before the increased mRNA expression levels of *FOXA2* by SFU conditions were identified to belong to the MEK-ERK and PI3K-AKT signaling pathways (figure 3(i)). Therefore, it is conclusive that the SFU culture conditions induced the expression of hepatocyte-related genes by activating various kinase signaling pathways.

# 3.4. Preparation of intestinal cells for the absorption aspect of NCCS

The human intestinal Caco-2 cell line represents the best available *in vitro* absorptive enterocyte. Usually

Caco-2 cells were differentiated into mature enterocytes in meshed transwell plates for 14-21 d until highly confluent (standard differentiation, ST). We used the same SFU protocol for Caco-2 cells as in HepaRG differentiation (supplementary figure 3). Morphology of Caco-2 cells under standard differentiation (ST) and SFU differentiation over 14 d is shown in figure 4(a). The TEER values of differentiated Caco-2 cells increased sharply from 7 d to 14 d post-initiation of differentiation, and that of cells under SFU differentiation were slightly higher than those of standard differentiation (ST) (figure 4(b)). We analyzed the mRNA expression of enterocyte markers, including CDX2, KRT20, ALPI, Villin, MDR1, GLUT2, GLUT5, SGLT1, and DPP4, in differentiated Caco-2 cells by qRT-PCR (figure 4(c)). Expression levels of all the markers were significantly increased in the differentiated Caco-2 cells compared to that in undifferentiated cells (CTL). Moreover, genes related to tight junction protein ZO-1 and gastrointestinal differentiation marker KRT20 were dramatically increased in Caco-2 cells differentiated using the SFU protocol compared to the mRNA levels under standard differentiation (ST) and undifferentiation (CTL) (figure 4(c)).

Differential genes was analyzed at 14 d, and data showed that genes associated with metabolism and transportation were upregulated, whereas those related to multicellular organ development and positive regulation of cellular processes were downregulated (supplementary figures 5(d)-(f)). Therefore, we used our SFU protocol for Caco-2 differentiation in subsequent experiments. CDX2, a regulator of intestinal gene expression, was expressed in the nuclei of differentiated Caco-2 cells while critical transporters, such as PEPT1 and MDR1, and tight junction markers ZO-1 and E-cadherin demonstrated increased expression in the membranes of differentiated Caco-2 cells (figure 4(d)). Fluorescence microscopic analysis of these differentiated Caco-2 cells revealed normal epithelial polarity, as indicated by a continuous linear distribution of tight junction proteins from the apical membrane with ZO-1 and restriction of F-actin to the basolateral membrane (figure 4(e)).

Permeability of the CTL and ST or SFU differentiated Caco-2-cell monolayers on mesh transwell inserts was monitored by the filtration of fluoresceinlabeled dextran. Fluorescein-labeled dextran was added into the upper chamber of the transwells (apical, A site), and fluorescence was quantified in samples from both the apical and lower chambers (basolateral, B site); animation is provided in figure 4(f). After 2 h, approximately 81% (ST) or 88% (SFU) of the dextran remained in the upper chamber in case of differentiated Caco-2 cells, whereas approximately 60% remained in the upper chamber in case of CTL cells. Moreover, after 3 h most of the dextran (>70%) passed to the basolateral side in the transwells of CTL



**Figure 3.** SFU induced activation of various kinases and expression of hepatocyte-enriched genes in HepaRG cells. (a), (b) Quantitative RT-PCR analysis of hepatocyte (a) or Bile-duct (b) related markers in undifferentiated HepaRG cells (CTL) after SFU at the indicated times. (c) Analysis of mRNA expression of hepatocyte or ER-stress related genes in HepaRG cells at indicated time point during SFU using RT-PCR. (d) Analysis of protein expression levels of hepatocyte or ER-stress related genes in HepaRG cells, cultured using conventional (CTL) or SFU conditions for 24 h by flow cytometry. Plots of the cell count versus DNA content (PI) are shown (e). Graph bars indicate G1, S, and G2/M phases of ST or SFU cultured cells (f). (g), (h) Analysis of cyclin D1/2, E1, A1, and B1 in mRNA level (g) by qRT-PCR and in the protein level (h) by western blotting of HepaRG cells, cultured using standard (ST) or SFU conditions for 24 h. (i) Analysis of the protein levels of Integrin-mediated MEK-ERK, AKT, JNK, and P38-MAPK signaling pathways by western blotting, using HepaRG cells at the indicated time points after SFU. (a), (b) and (g) Data are means  $\pm$  SEM (n = 10). \*p < 0.05.

cells, whereas approximately 68% (ST) or 80% (SFU) of dextran remained in the apical chamber in the case of differentiated Caco-2 cells (figure 4(g)). These results indicated that differentiated Caco-2 cells using the SFU protocol generated functional intestinal epithelial cells suitable for use in an *in vitro* absorption model.

### 3.5. Comparison of similarity of PK profiles (first-pass effect) of NCCS to in vivo results

First, we decided to validate NCCS as a reliable system to test the first-pass effect using two-dish channel before the expending to four-dish channel. To this end we used commercial drugs that have well-defined absorption and metabolism properties. In practice, acyclovir, ganciclovir, digoxin, and bretylium are classified as low-absorption, lowmetabolism, and low-BA drugs based on DrugBank (www.drugbank.ca) and previous studies by Lau et al [18], Mannery et al [19], Battino et al [20] Worthley et al [21], and Ochs et al [6]. A total of fifteen drugs categorized as low, medium, and high BA, as indicated in supplementary table S3, were used. Functional HepaRG and Caco-2 cells were prepared on an appropriate multi-insert dish as described in materials and methods and then loaded into NCCS containing the medium (25 ml/each unit). NCCS was operated for 48 h under controlled conditions, with a flow rate of  $2 \text{ ml min}^{-1}$  and shaking at 0.8 RPS.

Next, each drug was loaded into the small intestinal unit, and then samples were collected from the medium every 30 min up to a period of 5 h, and then after another 1 h for the analysis of early response of the drug (figure 5(a)). The samples were collected at 16, 24, and 48 h after their loading to analyze the late response. After that, we analyzed the relative concentration of the used parent compound by LC-MS/MS chromatography. The concentration of drugs in the medium increased from the start to maximum concentration (Cmax) and then decreased or remained at a relatively constant level at 48 h after inoculation. It means that NCCS showed PK profile that the drug could permeate through the intestine unit during the time until Cmax (Tmax) and disappeared from the medium by metabolic clearance of liver unit at a later time. Particularly, depend on the chemical, the time to reach Tmax was different from 2 to 6 h (Tmax) and the clearance ratio was also different after Tmax. PK profiles of three representative drugs, acyclovir, desipramine, and nifedipine, are shown in figure 5(b). Acyclovir revealed typical low absorption and low metabolism patterns, desipramine showed medium absorption and high metabolism, and high absorption and medium metabolism



permeability analysis. (g) Permeability analysis of Caco-2 cells differentiated using ST or SFU culture. Fluorescence-labeled dextran permeability was monitored for 180 min in both the upper and lower chambers of transwells containing differentiated Caco-2 cells and undifferentiated cells (CTL).

pattern appeared in nifedipine. These PK profiles of used fifteen reference drugs are presented in figure 5(c).

Further, the drugs were categorized into low, medium, and high absorption and metabolism based on their relative concentrations. Briefly, for absorption, the relative drug concentrations during the 6 h of analysis were categorized as follows; low absorption: <20%, medium absorption: 20%-50%, and high absorption: >50%. Similarly, for metabolism the relative drug concentrations were categorized as; high metabolism: <25%, medium metabolism: 20%-50%, and low metabolism: >50% (supplementary table S3). The data obtained from NCCS when compared with a human body revealed a high similarity of absorption (R = 0.7887) as well as metabolism (R = 0.748) respectively (figures 5(d) and (e)). The similarity of co-relation between the data from NCCS and human was obtained by best-fit sigmoidal functions [18]. Additionally, we compared in-silico predictor data (supplementary table S4) to the human body, and the similarity of absorption was R = 0.6089 (supplementary figure 6(a), left)

while that of metabolism was R = 0.144 (supplementary figure 6(a), right). Comparing *in-silico* predictor data to NCCS data, the similarity of absorption was R = 0.4324 (supplementary figure 6(b), left), and that of metabolism was R = -0.08 (supplementary figure 6(b), right). These data suggested that NCCS can predict the first-pass effect with relatively higher similarity to human data than *in-silico* predictor data. We believe that the experimental data from NCCS can help increase the similarity with *in-silico* predictor data.

## 3.6. Evaluation of differential metabolism of APAP across species by NCCS

In addition to PK, we attempted to show various applications of NCCS, such as investigation of species difference of drug liver metabolism and drug-induced toxicity. Animals, such as rodents, are commonly used in preclinical testing during drug development for predicting human responses to candidate chemicals. However, the existence of species differences, including the metabolic systems in the liver, pose critical limitations that decrease the success rate of



**Figure 5.** Pharmacokinetic and the first-pass effect (absorption and metabolism) assay using NCCS. (a) Illustration of the two-dish channel NCCS with Caco-2 and HepaRG cells to test first-pass effect. Caco-2 cells were cultured in bottom-meshed multi-insert culture dishes and HepaRG cells were cultured in side-meshed multi-insert culture dishes. The drugs were circulated from Caco-2 to HepaRG cells. (b) Pharmacokinetic profiles of acyclovir, desipramine, and nifedipine obtained from NCCS. The dots indicate the relative percentages of drugs by LC-MS/MS. (c) Drugs at 20 M (acyclovir, digoxin, theophylline, imipramine, phenytoin, ganciclovir, desipramine, nifedipine, nicardipine, chlorothiazide, propranolol, metoclopramide, acetaminophen, bretylium, and caffeine) were added to Caco-2 cells on multi-insert culture dish and circulated to the HepaRG cells. PK profiles of tested drugs (parent chemical) in NCCS over 48 h. (d) Correlation between certified human body absorption and absorption result from NCCS ( $C_{max}$ , %). (e) Correlation between certified human body metabolism (*in vivo* CL; ml min<sup>-1</sup> kg<sup>-1</sup>) and metabolism result from NCCS CL ( $\Delta C = C_{max} - C_{48 h}$ ). The correlation (R) of absorption and metabolism was calculated using Prism version 7.0 (GraphPad software) by best-fit sigmoid function.

drug development, with hepatotoxicity being a significant reason for withdrawing a drug from the market. To evaluate the differences in drug metabolism between humans and mice, we prepared mouse SIEP and AML12 in the appropriate multi-insert dish and then transferred them to NCCS. SIEP cells were cultured to enhance intestinal permeability for a further 8 d from the seeding and then validated for functionality by testing the expression of ZO-1 and the increase of TEER values (supplementary figures 7(a) and (b)). We also examined enhanced mRNA expression of enterocyte markers such as *Mrp1* and *P-gp* (supplementary figure 7(c)).

To analyze the function of tight junctions, we examined permeability using 3 KDa FITC-labeled Dextran. After 8 d of SIEP culturing, the dextran slowly passed through the monolayer compared to that after 1 d (undifferentiation) of SIEP culturing (supplementary figure 7(d)). AML12 cells were cultured with medium containing hepatic supplements (insulin, transferrin, and dexamethasone) until they were highly confluent with cuboidal shapes. We confirmed the hepatic functionality of AML12 cells using the protein expression and secretion of albumin and validated the induction of CYP2E1 protein by APAP treatment and ethanol (supplementary figures 7(e)–(g)). These cells were used as 'differentiated' cells, and in comparison, cells cultured without hepatic supplements were used as undifferentiated controls.

APAP is a nonsteroidal anti-inflammatory drug that is used worldwide. However, an overdose of APAP induces liver damage due to the toxic metabolite, N-acetyl-p-benzoquinoneimine (NAPQI) that is produced by CYP2E1 enzyme and GSH pathway, and plays a role in the elimination of NAPQI (figure 6(a)). To understand the mechanisms of metabolic differences of APAP in humans and mice, we compared CYP2E1, glutamate-cysteine ligase catalytic subunit (GCLC), glutathione peroxidase (GPX), glutathione reductase (GSR), superoxide dismutase 3 (SOD3), Glutathione S-transferase P1(GSTP1), Glutathione S-transferase M1 (GSTM1), and Glutathione S-transferase T1 (GSTT1) expression in differentiated HepaRG and AML12 cells. The expression levels of CYP2E1 were not changed, although the levels of GCLC, GPX, SOD3, GSTP1, GSTM1,

and GSTT1 increased in AML12 cells compared to that in HepaRG cells in transcriptional and translational levels (figures 6(b) and (c)). However, GSR levels increased in HepaRG cells compared to that in AML12 cells (figures 6(b) and (c)). This result suggested that consumption of free GSH, which plays a protective role against oxidative stress by NAPQI, occurred more rapidly in human cells than in mouse cells. To determine the quantitative differences of free GSH during APAP use in humans and mice, we measured the level of free GSH in HepaRG and AML12 cells after APAP treatment. Results demonstrated the free GSH concentration in human cells to be significantly reduced after treatment with APAP in a dosedependent manner compared to that in APAP-treated mouse cells (figure 6(d)). As shown in figure 6(e), phosphorylated JNK, an indicator for oxidative stress, was detected more strongly in human cells than in mouse cells. Mouse liver cells possess a more abundant GSH system than human liver cells; therefore, AML12 cells were more resistant to APAP than HepaRG cells in conventional culture (figure 6(f)). Next, we confirmed the species differences of APAP metabolism using mouse and human NCCS. The firstpass effect profiles of APAP from two NCCS (mouse and human) did not show significant differences at given concentration (2 mM APAP, data not shown). However, APAP-GSH, GSH adduct of NAPQI was significantly increased in mouse NCCS (figure 6(g), left), wherein 4-aminophenol, another toxic metabolite, was not significantly different (figure 6(g), right). After NCCS incubation, comparatively higher dead cell count was detected in APAP treated HepaRG than in AML12 cells (figure 6(h)), and active caspase-3 was also detected in APAP treated HepaRG cells (figure 6(i)) at 2 mM, a concentration, which is not effective in conventional cell culture conditions.

In addition to the oxidation pathway (Phase I), phase II conjugating enzymes were involved in metabolism up to 85%-90% of APAP. Therefore, we also compared phase II conjugating enzyme (glucuronidation- and sulfation-related enzyme) levels and their related metabolites (APAP-Gluc) in mouse to those of a human hepatocyte cells. The transcription level of enzymes related to glucuronidation pathways were more upregulated in AML12 than HepaRG cells (supplementary figures 8(a) and (b)), and APAP-Gluc was only detected in AML12 cells under both static culture and NCCS (supplementary figure 8(c)). Upon comparing the expression level of GSH based anti-oxidant system it was found that mouse cells possessed stronger than GSH based antioxidant system of human, but both appeared resistant to APAP at a high IC<sub>50</sub> of 30 mM and 10 mM, respectively in conventional culture conditions (figure 6(f)). However, NCCS showed more sensitivity to APAP than conventional cell culture conditions at given concentration (2 mM), and species difference

of APAP-GSH production, evidence of the existence of NAPQI.

## 3.7. Assessments of efficacy and renal toxicity using four-dish channel NCCS

Next, we aimed to provide the application of NCCS for the aspect of PD. Conceptually; NCCS was designed to use four channels with different cell sources. Indeed, we proved our concept by showing the efficacy of APAP and renal toxicity using four different cells, including intestine, liver, kidney, and macrophage. To conduct this test, we prepared kidney proximal tubule cells (HK-2) and monocyte (U937) cells in addition to SFU-differentiated HepaRG and Caco-2 cells (figure 7(a)). HK-2 cells possessed much higher level of gamma-GT activity compared to normal fibroblast (MRC5) and expressed specific markers for proximal tubules, such as SLC22A1, SLC22A2, SLC22A6, SLC22A8, SLC29A1, SLC15A1, MRP1, and MRP2 (figure 7(b)). These data suggested that HK-2 cells provided toxic responses to renal toxicity test. Next, U937 cells were differentiated into active macrophages by PMA for 3 d. Over 80% of cells expressed a macrophage marker (CD11b) and its related markers like CD11b, IL1b, IL6, IL8, IL10, TNFA, iNOS, and COX2 (figure 7(c)). Active macrophages also induced the secretion of prostaglandin E2 (PGE2), which causes the symptoms of inflammation (figure 7(c)). Used cells, including HK-2 and U937 cells, were not much sensitive to APAP concentration itself, under conventional cell culture condition (supplementary figure 9).

In four-dish channels NCCS, we observed the production of APAP metabolites, including 4aminophenol and APAP-GSH, to be same as that in two-dish channels system (figure 7(d)). It suggested that kidney and macrophages were not much effected to APAP metabolism. However we also observed inhibition of PGE2 secretion by APAP in a timedependent manner (figure 7(e)) and increased cell death and apoptosis by toxic metabolites (figure 7(f)). These data supported that HK-2 cells and U937 cells were sensitive to APAP metabolites in NCCS. Additionally, we observed increased apoptosis of HK-2 cells in response to APAP or APAP metabolites (figure 7(g)), and mRNA expression of kidney injury markers in these cells, such as LCN2, Clusterin, HAVCR1, and caspase-3, was significantly increased in NCCS in a time-dependent manner (figure 7(h)). These results suggested NCCS as a possible platform to assess both efficacy and toxicity, simultaneously.

### 4. Discussion

PK parameters represent the absorption, metabolism, distribution, and elimination (ADME) of chemical compounds *in vivo*, and are directly related to toxicity and efficacy [22, 23]. Recently, various cell culture



24 h by EZ-cytoX assay at conventional culture condition. Data are means  $\pm$  SD (n = 5). \*p < 0.05. (g) Detection of APAP-GSH and 4-aminophenol of 2 mM APAP treated HepaRG and AML12 cells by LC-MS/MS at indicated time points in NCCS. Data are means  $\pm$  SD (n = 5). \*p < 0.05. (h), (i) Analysis of cell survival using a live/dead cell stain (h) and the expression of caspase-3 and p-JNK protein (i) of 2 mM APAP-treated HepaRG and AML12 cells in NCCS.

platforms have been proposed to replicate in vivo environments with respect to complexity, organ networking, and nutrition circulation [7, 17]. Organ-ona-chip has been considered to be the best tool available for mimicking in vivo condition; however, its small scale of analysis and challenging conditions of cell survival are considered to be problematic limitations by many cell biologists [24]. Thus, there remains a need for a platform that functions more closely to real cell culture condition to provide optimal conditions of cell growth and recapitulate in vivo environments, as well. Based on this notion, we designed and fabricated the NCCS platform, which can provide real cell culture conditions and mimic in vivo environments, along with networking, and circulation. NCCS is a relatively macroscale system compared to chip-based devices, and involves cells supplied with adequate amounts of media; double-layered dishes used for NCCS can be modulated to control oxygen gradients through air filters. Due to the macroscopic scale of NCCS, a sufficient number of cells and supernatants (media) could be collected for the analysis of proteins, RNA, and DNA at the cellular level, as well as the area under the plasma drug concentration-time

curve (AUC) of the drug could be determined after the test.

NCCS does required well-differentiated cells to mimic organ-networking. A HepaRG differentiation protocol has been developed using DMSO; however, it requires a relatively long time for the cells to differentiate into functional hepatocytes. Therefore, a more effective and reproducible differentiation protocol would be required to acquire functional hepatocytes. In the current study, we found SFU [16], using rotational cell-culture techniques, to promote hepatic differentiation of HepaRG cells. The protocol revealed, up-regulation of hepatocyte markers, whereas the expression of biliary markers was down-regulated. This suggested that SFU selectively promotes hepatocyte differentiation. In a previous report, shearstress was shown to cause flow-induced hepatocyte differentiation; however, the mechanism remained unexplored [25]. The present study revealed that SFU activated MEK-ERK signaling in HepaRG cells quite early in the process, resulting in the sequential activation of FOXA2, although the cell cycle was also inhibited. Earlier reports have also shown that the shearstress inhibits cell growth, along with suppression of



**Figure 7.** The assessment of efficacy and drug-induced renal toxicity of APAP using NCCS. (a) Illustration of the four-dish channel NCCS with Caco-2, HepaRG, HK-2, and U937 cells. APAP was circulated from Caco-2 to HepaRG, HK-2, and U937 cells. (b) Functional analysis of HK-2 cell using gamma-GT activity compare to normal fibroblasts, MRC-5 (left). Analysis of the mRNA expression of kidney transporters genes (*SLC22A1, A2, A6, A8, SLC29A1, SLC15A1, SLC15A2, MRP1*, and *MRP2*) by qRT-PCR in HK-2 cells compared to those in MRC-5 cells (right). (c) FACS analysis of expression of CD11b in PMA-treated U937 for 72 h (left). Analysis of the mRNA expression of active macrophage marker genes (*CD11b, IL1b, IL6, IL8, TNFA, iNOS,* and *COX2*) by qRT-PCR in PMA-treated U973 compared to that in untreated U937 cells (control) (middle). The concentration of prostaglandin E2 (PGE2) in untreated (control) and PMA-treated U937 cells (right). Data are means  $\pm$  SD (n = 5). \*p < 0.05. (d) Detection of 4-aminophenol and APAP-GSH by LC-MS/MS after treatment with 1 mM APAP at indicated time points (hour) in NCCS. (e) Analysis of the concentration of prostaglandin E2 (PGE2) at 0, 6, 24, and 48 h after APAP treatment in NCCS. Data are means  $\pm$  SD (n = 5). \*p < 0.05. (f) Cell live/dead staining using HK-2 and U937 cells 48 h post-treatment [left]. Western blot analysis of the activation of caspase-3 signaling (right). (g) Analysis of apoptosis of HK-2 cells using MUSE cell analyzer at 6, 24, and 48 h post-treatment in NCCS. (h) mRNA level of renal toxic marker genes *LCN1, CLU, HAVCR1*, and *CASP3* in APAP treated HK-2 cells compared to that in untreated cells (CTL) at 24 and 48 h after NCCS incubation. Data are means  $\pm$  SEM (n = 10). \*p < 0.05.

cell transition from G1 to S phase of the cell cycle in endothelial cells [26], and at the same time, activated MEK-ERK signaling helps cell-survival by modulating ECM under a non-attached environment. Previously, we had shown that SFU modulated ECM compartments and epithelial-mesenchymal transition (EMT) of Huh-7 liver cancer cells [16]. However, the present study revealed the up-regulation of FOXA2 by SFU was recently investigated in healthy hepatocyte-like HepaRG cells. FOXA2 was found to be involved in tissue regionalization and act as a master controller of liver development [27]. FOXA2, as a transcription factor, binds to its target gene promoter and regulates the level of mRNA expression. C/EBP- $\alpha$ , C/EBP- $\beta$ , USF2, HNF4 $\alpha$ , and CREBH genes are transcriptionally regulated by FOXA2 [27]. Owing to the lack of available human fetal tissues, the specific role of FOXA2 in human embryonic development has not been determined yet, although studies using rodent

embryos and a human ES cell model have supported the role of FOXA2 in liver development [27].

Poor PK properties, such as low BA, account for nearly 50% of the clinical failures during drug development and testing. While most PK tests are conducted using rodents, they include different drugs to utilize the systems. For example, rodent models demonstrate different expression levels and activities of some CYP isoforms, and that affects PD validation. Drug-induced toxicity could not be tested by non-integrated single cell. Therefore, a system and a protocol were developed to acquire accurate data that would reflect the human response more accurately. We designed and fabricated the NCCS using human cells. Our results using the NCCS showed similarity to more than 70% of the results from the human body with regard to absorption and metabolism. Notably, drugs that were categorized into extremely low or extremely high absorption and metabolism

group were more similar than those classified into the medium category, and we speculated that the remaining 30% discrepancy was due to the absence of an elimination component of the NCCS such as urine or sweat. To acquire more accurate data from the NCCS, an elimination component must be added, since in vivo BA is the product from systematic harmonization of absorption, metabolism, and elimination. Additionally, using more functional and highly differentiated cells, such as organoids, rather than cell lines may help to increase the accuracy of NCCS. There is no doubt that Caco-2 and HepaRG cell lines are suitable models for permeability and metabolism assessment in vitro; however, these cell lines were isolated from cancerous tissues, which means that a large number of mutations have occurred during assessment [28]. Although it is not guaranteed that organoids are more useful than established cell lines, well-differentiated organoids could be more representative of healthy tissue development [29, 30].

Drugs are metabolized by various liver mechanisms, and some of them result in hepatotoxicity. Specifically, the production of toxic metabolites is the most frequent cause of hepatotoxicity. APAP is one of the best known hepatotoxic drugs that cause hepatotoxicity via the toxic metabolite NAPQI. NAPQI converted rapidly into GSH-conjugating form, APAP-GSH, in vivo, and eliminate it from the body; therefore, an overdose of APAP results in depletion of GSH in the liver, which increases oxidative stress in the liver. Therefore, when considering the hepatotoxicity of APAP, various parameters must be considered [26]. Interestingly, HepaRG cells consumed more copious amounts of GSH than the mouse hepatocyte cell line AML12 when they metabolized APAP; therefore, the free-GSH concentration in HepaRG was significantly reduced after treatment with APAP compared to that in AML12 cells. It is suggested that AML12 cells are less sensitive to hepatotoxicity than HepaRG cells, since they efficiently metabolized APAP by up-regulating GSH metabolism enzymes in conventional cell culture condition. However, both mice and humans were sensitive at lower concentration of APAP in NCCS than conventional cell culture conditions. We speculated that NCCS showed more similar result in vivo than conventional cell culture since the live mouse has been known to be sensitive to APAP.

APAP has been reported to reveal drug-induced renal toxicity [31]. While renal tubular cells, HK2, were not much sensitive to APAP itself in conventional cell culture condition, four-dish channels NCCS showed the drug to induce renal cell toxicity like *in vivo*. It suggested that the assessment of renal toxicity requires an integrated organ system. Here, we showed the possibility and qualification of an *in vivo* mimicking cell culture system like NCCS to be used in drug development. We expect that a furtherdeveloped system, using organoids, would provide even better information than this system regarding human similarity.

### 5. Conclusion

Here, we fabricated NCCS for conducting PK and PD assays *in vitro*. The NCCS was constructed by all-inone system using a micro piezo pump, weight sensor, double-layered cell-culture ware, and control panel, which compare to separated system such as organ chips. Additionally, we suggested a new effective cell differentiation protocol using spheroid forming unit (SFU) platform. We founded that SFU promoted the hepatic differentiation of HepaRG and strengthen the tight junction of Caco-2 cells. Finally, we assessed first-pass PK of fifteen reference drugs and showed the similarity of the data from NCCS between data from human body. The NCCS provided absorption and metabolism data similar to >70% of the human data.

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### Author contributions

Conceived and designed the experiments: JHL(A) and CRJ. Performed the experiments: KHN, HMK, HSC, JYL, DKL and DSK. Analyzed the data: KSC, SJO, MYS, JHL(A), and CRJ. Contributed reagents and materials: DHK, MK, KSC and JHL. Wrote the paper: JHL(A) and CRJ.

### **Conflict of interest**

The authors declare that they have no competing financial interests.

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### References

- Yanagita T 1975 Some methodological problems in assessing dependence-producing properties of drugs in animals *Pharmacol. Rev.* 27 503–9
- [2] Kennedy T 1997 Managing the drug discovery/development interface Drug Discov. Today 2 436–44

- [3] Sietsema W 1989 The absolute oral bioavailability of selected drugs Int. J. Clin. Pharmacol. Ther. Toxicol. 27 179–211
- [4] Kirkpatrick C J, Fuchs S, Hermanns M I, Peters K and Unger R E 2007 Cell culture models of higher complexity in tissue engineering and regenerative medicine *Biomaterials* 28 5193–8
- [5] Guillouzo A, Corlu A, Aninat C, Glaise D, Morel F and Guguen-Guillouzo C 2007 The human hepatoma HepaRG cells: a highly differentiated model for studies of liver metabolism and toxicity of xenobiotics *Chem. Biol. Interact.* 168 66–73
- [6] Ochs H R, Greenblatt D J, Bodem G and Harmatz J S 1978 Dose-independent pharmacokinetics of digoxin in humans Am. Heart J. 96 507–11
- [7] Skardal A, Shupe T and Atala A 2016 Organoid-on-a-chip and body-on-a-chip systems for drug screening and disease modeling *Drug Discov. Today* 21 1399–411
- [8] Knöspel F, Jacobs F, Freyer N, Damm G, De Bondt A, Van den Wyngaert I, Snoeys J, Monshouwer M, Richter M and Strahl N 2016 In vitro model for hepatotoxicity studies based on primary human hepatocyte cultivation in a perfused 3D bioreactor system Int. J. Mol. Sci. 17 584
- [9] Uzel S G, Platt R J, Subramanian V, Pearl T M, Rowlands C J, Chan V, Boyer L A, So P T and Kamm R D 2016 Microfluidic device for the formation of optically excitable, three-dimensional, compartmentalized motor units *Sci. Adv.* 2 e1501429
- [10] Vernetti L, Gough A, Baetz N, Blutt S, Broughman J R, Brown J A, Foulke-Abel J, Hasan N, In J and Kelly E 2017 Functional coupling of human microphysiology systems: intestine, liver, kidney proximal tubule, blood-brain barrier and skeletal muscle Sci. Rep. 7 42296
- [11] Edington C D, Chen W L K, Geishecker E, Kassis T, Soenksen L R, Bhushan B M, Freake D, Kirschner J, Maass C and Tsamandouras N 2018 Interconnected microphysiological systems for quantitative biology and pharmacology studies *Sci. Rep.* 8 1–18
- [12] Sambuy Y, De Angelis I, Ranaldi G, Scarino M, Stammati A and Zucco F 2005 The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics *Cell Biol. Toxicol.* 21 1–26
- [13] Hidalgo I J, Raub T J and Borchardt R T 1989 Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability *Gastroenterology* 96 736–49
- [14] Kanebratt K P and Andersson T B 2008 Evaluation of HepaRG cells as an *in vitro* model for human drug metabolism studies *Drug Metab*. *Dispos.* 36 1444-52
- [15] Freund J B, Goetz J G, Hill K L and Vermot J 2012 Fluid flows and forces in development: functions, features and biophysical principles *Development* 139 1229–45
- [16] Jung H-R, Kang H M, Ryu J-W, Kim D-S, Noh K H, Kim E-S, Lee H-J, Chung K-S, Cho H-S and Kim N-S 2017 Cell

spheroids with enhanced aggressiveness to mimic human liver cancer *in vitro* and *in vivo Sci. Rep.* **7** 10499

- [17] Kimura H, Ikeda T, Nakayama H, Sakai Y and Fujii T 2015 An on-chip small intestine–liver model for pharmacokinetic studies J. Lab. Autom. 20 265–73
- [18] Lau Y Y, Chen Y-H, Liu T-T, Li C, Cui X, White R E and Cheng K-C 2004 Evaluation of a novel in vitro Caco-2 hepatocyte hybrid system for predicting *in vivo* oral bioavailability *Drug Metab. Dispos.* **32** 937–42
- [19] Mannery Y O, Ziegler T R, Park Y and Jones D P 2010 Acetaminophen elimination half-life in humans is unaffected by short-term consumption of sulfur amino acid-free diet J. Pharmacol. Exp. Ther. 333 948–53
- [20] Battino D, Croci D, Mamoli D, Messina S and Perucca E 2004 Influence of aging on serum phenytoin concentrations: a pharmacokinetic analysis based on therapeutic drug monitoring data *Epilepsy Res.* 59 155–65
- [21] Worthley L and Holt A 1999 Digoxin in the critically ill patient *Crit. Care Resusc.* **1** 252
- [22] Wang J and Urban L 2004 The impact of early ADME profiling on drug discovery and development strategy *Drug Discov. World* 5 73–86
- [23] Löbenberg R and Amidon G L 2000 Modern bioavailability, bioequivalence and biopharmaceutics classification system. New scientific approaches to international regulatory standards *Eur. J. Pharm. Biopharm.* **50** 3–12
- [24] Sbrana T and Ahluwalia A 2012 New Technologies for Toxicity Testing (Berlin: Springer) pp 138–53
- [25] Rashidi H, Alhaque S, Szkolnicka D, Flint O and Hay D C 2016 Fluid shear stress modulation of hepatocyte-like cell function *Arch. Toxicol.* **90** 1757–61
- [26] Muldrew K L, James L P, Coop L, McCullough S S, Hendrickson H P, Hinson J A and Mayeux P R 2002 Determination of acetaminophen-protein adducts in mouse liver and serum and human serum after hepatotoxic doses of acetaminophen using high-performance liquid chromatography with electrochemical detection *Drug Metab. Dispos.* **30** 446–51
- [27] Lau H H, Ng N H J, Loo L S W, Jasmen J and Teo A K K 2017 The molecular functions of hepatocyte nuclear factors in and beyond the liver J. Hepatol. 68
- [28] Roerink S F, Sasaki N, Lee-Six H, Young M D, Alexandrov L B, Behjati S, Mitchell T J, Grossmann S, Lightfoot H and Egan D A 2018 Intra-tumour diversification in colorectal cancer at the single-cell level *Nature* 556 457
- [29] Hubatsch I, Ragnarsson E G and Artursson P 2007 Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers *Nat. Protoc.* 2 2111
- [30] Fatehullah A, Tan S H and Barker N 2016 Organoids as an *in vitro* model of human development and disease *Nat. Cell Biol.* 18 246
- [31] Mazer M and Perrone J 2008 Acetaminophen-induced nephrotoxicity: pathophysiology, clinical manifestations, and management J. Med. Toxicol. 4 2–6