Letter

Requirements for human iPS cell-derived hepatocytes as an alternative to primary human hepatocytes for assessing absorption, distribution, metabolism, excretion and toxicity of pharmaceuticals

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ABSTRACT — Predicting drug-induced liver injury is still a big challenge for the research and development of pharmaceuticals. Primary human hepatocytes (PHH) are the gold standard cell sources for examining drug metabolism, drug-drug interaction (DDI) and hepatotoxicity in humans *in vitro*. However, their supply does not meet the demand of laboratories sufficiently, and only a limited number of lots of PHH are commercially available. Recently, human iPS cell-derived hepatocytes have been reported and are anticipated to be used as an alternative to PHH. However, drug metabolizing activities of these human iPS-derived hepatocytes have not been well characterized and quantitative target values for PHH alternatives remain unknown. In this study, we collected 179 data sheets of commercially available PHH lots from 4 vendors to clarify the characteristics of PHH in drug metabolism and DDI. We identified the most frequently observed value ranges (MFRs) of the activities of major drug metabolizing enzymes (CYP3A4/5, CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, UGT, and SULT) and drug transporters. Moreover, we also identified MFRs of fold changes of CYP inductions by each typical inducer both in the mRNA levels and the enzyme activity levels of CYP1A2, CYP2B6, and CYP3A. We suggest that these MFRs are the first milestone to develop and evaluate human iPS cell-derived hepatocytes as alternatives to PHH in pharmaceutical research for assessing absorption, distribution, metabolism, excretion, and toxicity.

Key words: Human iPS cell-derived hepatocytes, Hepatotoxicity, Drug metabolism, Cytochrome P450

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INTRODUCTION

Drug induced liver injury (DILI) is one of the major causes of attrition or withdrawal of pharmaceuticals (Chen *et al*., 2011). The prediction of the risks of DILI at the preclinical stage has been a big challenge in the safety assessment of pharmaceuticals. However, there are many questions that remain to be solved. For instance, it is difficult to predict the incidence of DILI in clinical trials from the results of the preclinical studies when the metabolite profiles of the compounds are different between humans and experimental animals (Bogaards *et al*., 2000). Many *in vitro* absorption, distribution, metabolism, excretion, and toxicity (ADMET) test methods have been widely used to predict the drug metabolism or the risk of DILI in humans at the preclinical stage using liver derivatives, such as microsomes and S9, and primary human hepatocytes (PHHs) (Xu *et al*., 2008; Di *et al*., 2012). However, PHHs are expensive and the number of PHH donors available is limited. The supply of a single PHH lot also has limitations because PHHs are unable to proliferate and be propagated with their hepatic functions. Moreover, PHHs have very large inter-lot differences so that the ADMET researchers cannot directly compare the results from them.

Other reasons for the difficulty of the DILI prediction in preclinical studies are the individual differences due to gene polymorphisms (Kurose *et al*., 2012; Pressiner *et al*., 2013; Zanger and Schwab, 2013) and PHHs obtained from donors who have rare single nucleotide polymorphisms (SNPs) are difficult to obtain, further complicating the prediction of idiosyncratic DILI. It is clear to see why the guideline for the evaluation of drug-drug interactions from the FDA recommends that multiple PHH lots be examined (FDA, 2012).

Recently, methods for differentiation to hepatocyte-like cells from human embryonic stem cells (hESC) or human induced pluripotent stem cells (hiPSC) have been reported (Shiraki *et al.*, 2011; Si-Tayeb *et al.*, 2010; Sullivan *et al.*, 2010; Takayama *et al.*, 2012), and some of them are commercially available. HiPSC-derived hepatocytes especially, are expected to be utilized in the future to predict clinical DILI, because hiPSC, which are easily established from somatic cells, proliferate repeatedly. If large amounts of hiPSC-derived hepatocytes can be produced inexpensively, they could be available for high throughput drug screening. For these reasons, hiPSC-derived hepatocytes, which contain hepatic functions similar to those of PHHs, have been anticipated to be utilized as reproducible alternatives to PHHs in pharmaceutical research and development, especially in ADMET eval-

uations (reviewed in Mann, 2015; Sauer *et al*., 2014; Schwartz *et al*., 2014).

However, no quantitative target values of the hepatic functions of hiPSC-derived hepatocytes, as alternatives to PHHs, have been indicated for pharmaceutical ADMET research. Because different PHH lots have been used as controls in several studies on hiPSC-derived hepatocytes (Shiraki *et al*., 2011; Takayama *et al*., 2012; Ulvestad *et al*., 2013), it is difficult to directly compare the hepatic functions of the reported hiPSC-derived hepatocytes from different papers. As members of the Consortium for Safety Assessment using Human iPS Cells (CSAHi), we collected the data from the certificates of analyses (CoAs) of commercially available PHHs provided by the vendors, and examined them to identify the requirements for hiPSC-derived hepatocytes as alternatives to PHHs. We determined the distributions of the activities of the major cytochrome P 450 (CYP) isoforms, the phase 2 enzymes (UGT and SULT), and CYP induction and transporters, which have important roles in the drug metabolism and drug-drug interactions (DDI) in PHHs. From our results, most frequently observed value ranges (MFRs) were identified in each evaluation point. We think that these MFRs are the first target values of the hiPSC-derived hepatocytes, which are suitable for alternatives to PHHs for pharmaceutical ADMET studies. Moreover, we will discuss the future directions of hiPSC-derived hepatocytes in pharmaceutical research and development, based on our literature search.

MATERIALS AND METHODS

Data sources

We used 179 CoAs (163 single donors and 16 pooled donors) from 4 vendors, which sell cryopreserved PHHs in Japan (Table 1). We collected the basic information of donors including sex, race, age, drinking, smoking, and drug history. We analyzed the distributions of the activities of major CYP isoforms (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4/5, and CYP2E1), UGT, and SULT. CYP induction activities (CYP1A2, CYP2B6, and CYP3A4/5) were analyzed for both the mRNA and the enzymatic activity levels. The detailed methods for the evaluation of the transporter activities are described below. The test conditions of each vendor are summarized in Tables S1-S3 (see Supplemental data).

Evaluation of the transporter activities

Since velocity of the transport process (Vmax) in the collected CoA is estimated at various substrate concentrations (0.1, 1, 2, 25 and 250 μM) among different ven-

Table 1. Number of primary human hepatocyte lots.

Vendor	Number of lots
A (single donor)	64
B (single donor)	50
C (single donor)	25
D (single donor)	24
B (pooled)	16
Total	179

dors and velocities are depended on substrate concentration, the velocities collected from CoAs needed to be normalized for direct comparison. Velocities of taurocholate, estrone-3-sulfate, cholecystokinin octapeptide (CCK8), estradiol 17β-(D)-glucuronide (E2-17G), and MPP⁺ at 0.1, 1, 2, 25, and 250 μM were estimated using the Michaelis Menten equation and respective parameters (Tables S4 and S5). Corrected velocities (Vmax) at a 1 μM substrate concentration were calculated based on the estimated ratio of velocity at different substrate concentrations and used for direct comparison.

Michaelis Menten equation:

$$v = \frac{Vmax \cdot S}{Km + S}$$

where v is the uptake velocity of the substrate (pmol/min/mg), S is the substrate concentration in the medium (μM), Km is the Michaelis constant (μM), and $Vmax$ is the maximum uptake rate (pmol/min/mg).

Statistical analyses

Data are presented as the mean + standard deviation (SD) of each hepatocyte group in log scale (Figs. 1, 2 and 5). Statistical significance was determined by a Student's t test (Microsoft Excel).

RESULTS

Basic information of donors

Donor's basic information is summarized in Table S6. The number of males and females was almost the same. Most of donors were Caucasians and 90.7% (147/163) over 20 years of age. Donors who smoke, drink alcohol, or use drugs were fewer than abstainers.

Activities of drug metabolizing enzymes

The number of analyzed PHH lots, CYP isoforms or phase 2 enzymes, their substrates, and reactions are shown in Table S7. Each enzymatic activity was simi-

lar between single donor hepatocytes and pooled hepatocytes (Fig. 1). We also compared the enzymatic activities between hepatocyte suspension and plated hepatocytes to clarify the influence of the culture conditions (Fig. 2). Plated hepatocytes showed less enzymatic activity than hepatocyte suspensions, except for the SULT activity. The distributions of the activities of CYP isoforms, UGT, and SULT are shown in Fig. 3. On the basis of these distribution data, we identified the MFRs of drug metabolizing enzyme activities in PHHs (Table 2).

CYP1A2

The CYP1A2 activity was evaluated by the acetaminophen production from phenacein O-dealkylation for all 4 vendors. The MFR was 20-30 pmol/million cells/min (Fig. 3A).

CYP2C9

The CYP2C9 activity was evaluated by diclofenac 4'-hydroxylation for vendors A and B. The MFR was 100-200 pmol/million cells/min (Fig. 3B). Tolbutamide was used as the substrate for vendor C (data not shown). No data on the CYP2C9 activity were provided from vendor D.

CYP2C19

The CYP2C19 activity was evaluated by S-mephenytoin 4'-hydroxylation for all 4 vendors. The MFR was 1-10 pmol/million cells/min (Fig. 3C).

CYP2D6

The CYP2D6 activity was evaluated by dextromethorphan O-demethylation for all 4 vendors. The MFR was 10-20 pmol/million cells/min (Fig. 3D).

CYP3A4/5

The CYP3A4/5 activities were evaluated by midazolam 1'-hydroxylation for vendors A, B, and C, and by testosterone 6β-hydroxylation for vendors A, B, and D. The MFRs were 20-30 pmol/million cells/min (Fig. 3E, midazolam) and 50-100 pmol/million cells/min (Fig. 3F, testosterone), respectively.

CYP2E1

The CYP2E1 activity was evaluated by chlorzoxazone 6-hydroxylation for vendors A and B. The MFR was 50-100 pmol/million cells/min (Fig. 3G). No data on the CYP2E1 activity were provided from vendors C and D.

UGT

The UGT activity was evaluated by hydroxycoumarin
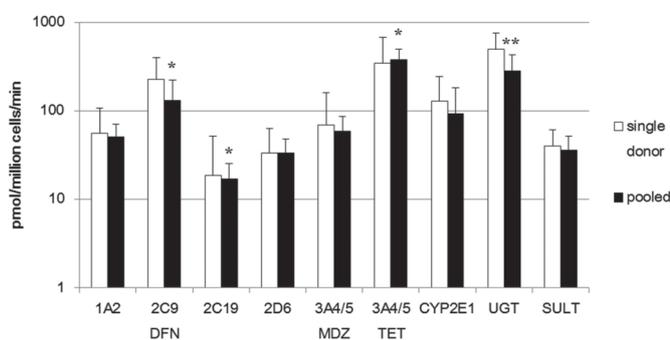
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Fig. 1. Comparison of drug metabolizing activities between single donor hepatocytes and pooled hepatocytes. Drug metabolizing activity of each enzyme was compared to PHHs from a single donor (open) and multiple donors (solid). Data shown are the mean + standard deviation (SD). *, $p < 0.05$; **, $p < 0.01$.
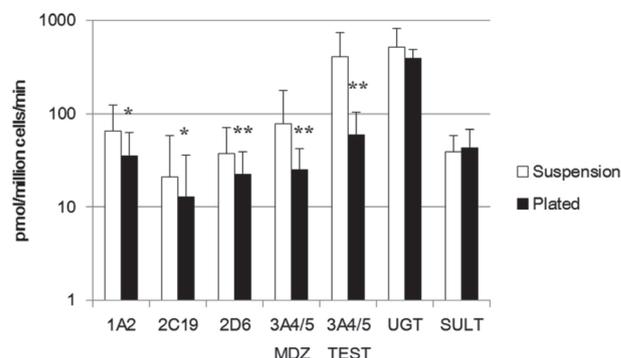

Fig. 2. Comparison of drug metabolizing activities between hepatocyte suspension and plated hepatocytes. Drug metabolizing activity of each enzyme was compared to PHHs from a single donor which were cultured in suspension (open) and plated (solid). Data shown are the mean + SD. *, $p < 0.05$; **, $p < 0.01$.

7-glucuronidation for vendors A, B, and C. The MFR was 300-500 pmol/million cells/min (Fig. 3H). Acetaminophen was used as the substrate for vendor D (data not shown).

SULT

The SULT activity was evaluated by hydroxycoumarin 7-sulfonation for vendors A, B, and C. The MFR was 20-30 pmol/million cells/min (Fig. 3I). Acetaminophen was used as the substrate for vendor D (data not shown).

CYP induction activities

The CYP inducers induced CYP isoforms; their substrates and the number of analyzed PHH lots are shown in Table S8. The distributions of the CYP induction activities are shown in Fig. 4. On the basis of these distribution data, we identified the MFRs of the CYP induction activities in PHHs (Table 3).

CYP1A

The CYP1A induction was evaluated by the increase of the metabolizing activity of phenacetin and the *CYP1A* mRNA after omeprazole treatment for all 4 vendors. The MFRs of the CYP1A induction rate were 5-35 times for the enzymatic activity level (Fig. 4A), and 5-10 times for the mRNA level (Fig. 4B).

CYP2B6

The CYP2B6 induction was evaluated by the increase of the metabolizing activity of bupropion and the *CYP2B6* mRNA after phenobarbital treatment for all 4 vendors. The MFRs of the CYP1A induction rate were 4-12 times

for the enzymatic activity level (Fig. 4C), and 4-18 times for the mRNA level (Fig. 4D).

CYP3A4/5

The CYP3A4/5 induction was evaluated by the increase of the metabolizing activity of testosterone and the *CYP3A4/5* mRNA after rifampicin treatment for all 4 vendors. The MFRs of the CYP1A induction rate were 5-10 times for the enzymatic activity level (Fig. 4E), and 5-20 times for the mRNA level (Fig. 4F).

Transporter activities

The influx transporter activities for intake of taurocholate, estrone-3-sulfate, CCK8, E2-17G, and MPP$^+$ in PHHs, which were evaluated for multiple vendors, were analyzed (Table S9). However, the culture conditions of PHHs were different among vendors when evaluating the transporter activities. Plated hepatocytes were used by vendors A and C, while hepatocyte suspension was used by vendors B and D (Fig. 5). We analyzed data from 66 single donor hepatocytes (Fig. 6) and identified the MFR of the transporter activity for each substrate in PHHs (Table 4).

DISCUSSION

Cryopreserved PHHs have been widely used in pharmaceutical *in vitro* ADMET studies for clinical prediction. However, there are two major problems in the use of PHHs in drug screening. One is the limited supply of the same lot because PHHs are unable to be proliferated with their hepatic functions. The other is the large varia-

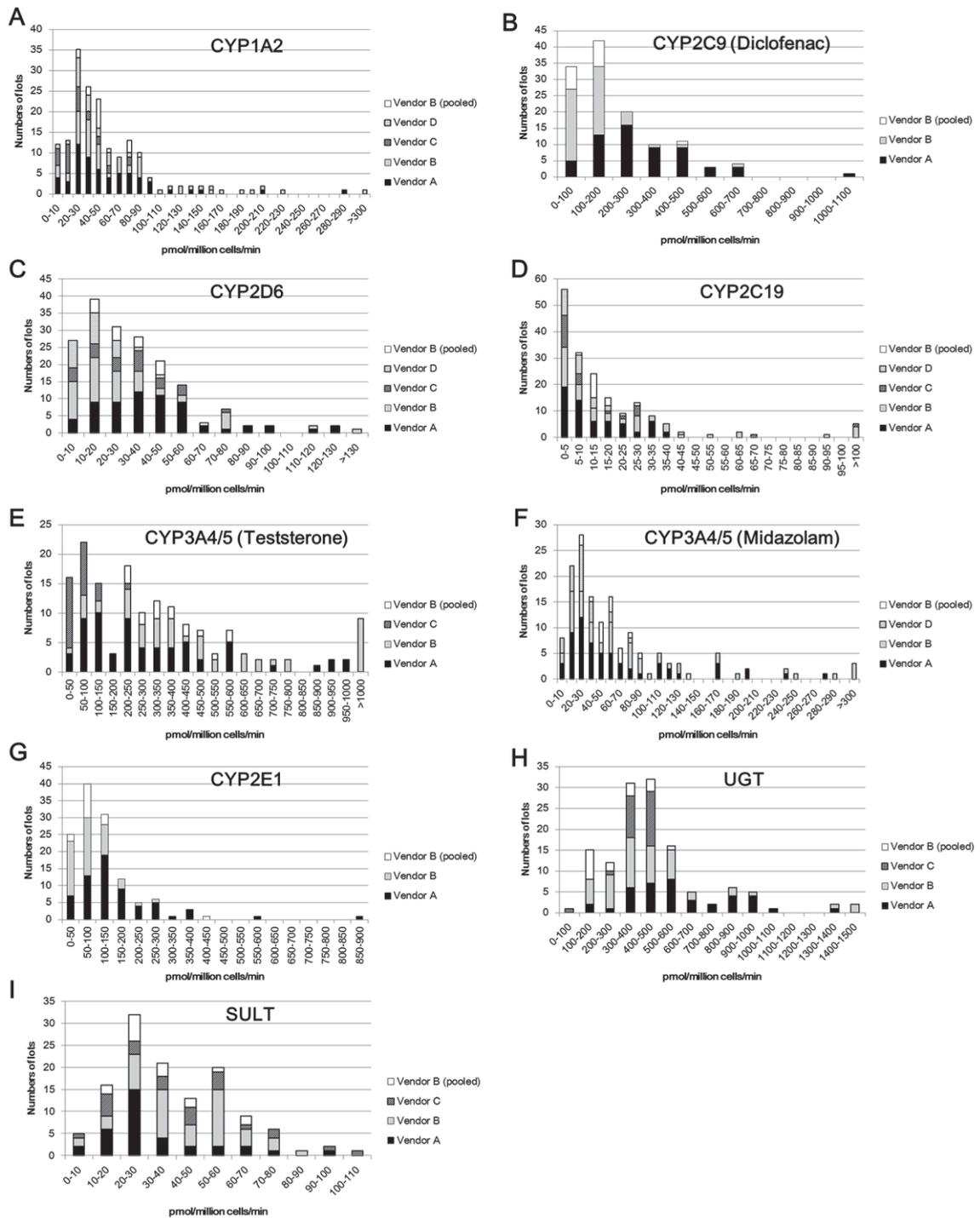Requirements for human iPS cell-derived hepatocytes

Fig. 3. Distributions of drug metabolizing activity of each enzyme in PHH. Drug metabolizing activity of each enzyme in both hepatocyte suspension and plated hepatocyte and numbers of lots is shown. (A) CYP1A2 activity was measured by phenacetin O-dealkylation. (B) CYP2C9 activity was measured by diclofenac 4'-hydroxylation. (C) CYP2C19 activity was measured by S-mephenytoin 4'-hydroxylation. (D) CYP2D6 activity was measured by dextromethorphan O-demethylation. (E and F) CYP3A/5 activity was measured by testosterone 6β-hydroxylation and midazolam 1'-hydroxylation. (G) CYP2E1 activity was measured by chlorzoxazone 6-hydroxylation. (H and I) UGT and SULT activities were measured by 7-hydroxycoumarin glucuronidation and sulfonation, respectively.

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Table 2. MFR of drug metabolizing activities for each enzyme.

Enzyme	Substrate and reaction	MFR (pmol/million cells/min)
CYP1A2	Phenacetin *O*-dealkylation	20-30
CYP2C9	Diclofenac 4'-hydroxylation	100-200
CYP2C19	*S*-Mephenytoin 4'-hydroxylation	1-10
CYP2D6	Dextromethorphan *O*-demethylation	10-20
CYP3A4/5	Midazolam 1'-hydroxylation	20-30
CYP3A4/5	Testosterone 6β-hydroxylation	50-100
CYP2E1	Chlorzoxazone 6-hydroxylation	50-100
UGT	7-Hydroxycoumarin-Glu	300-500
SULT	7-Hydroxycoumarin-Sul	20-30

MFR: most frequently observed value range.
Glu: Glucuronidation
Sul: Sulfonation

Table 3. MFR of CYP induction activities in PHH.

Enzyme	Inducer	MFR Enzyme activity (fold change)	MFR mRNA expression (fold change)
CYP1A2	Omeprazole	5-35	5-10
CYP2B6	Phenobarbital	4-12	4-18
CYP3A4/5	Rifampicin	5-10	5-20

MFR: most frequently observed value range.

tions between lots due to their different genetic and environmental backgrounds. HiPSC-derived hepatocytes have been anticipated to be used in *in vitro* ADMET studies as alternatives to PHHs because researchers are able to obtain the hiPSC-derived hepatocytes from the same origin repeatedly (Holmgren *et al.*, 2014; Lu *et al.*, 2015; Sirenko *et al.*, 2014). PHHs were often used as controls in the studies of hiPSC-derived hepatocytes. However, it is also difficult to directly compare the hepatic functions of the hiPSC-derived hepatocytes from different papers or laboratories because of the lot-to-lot variations of PHHs as described above (Shiraki *et al.*, 2011; Takayama *et al.*, 2012; Ulvestad *et al.*, 2013).

We collected CoAs of commercially available PHHs from 4 vendors and showed the distributions of the drug metabolizing activities, CYP induction activities, and transporter activities (Figs. 3, 4 and 6). We confirmed that there were large differences between lots in the levels of the drug metabolizing, CYP induction, and transporter activities. Four vendors adopted different methods to each other and examined the hepatic functions of PHHs (Tables S1-S3). The activities of drug metabolizing enzymes and transporters were lower in plated hepatocytes than in hepatocyte suspensions. In CYP induction activities, we thought that the different concentrations of

the inducers do not affect the results because there were few correlations between the concentration of the inducers and the distributions of the induction ratios for each vendor.

In this study, we identified the MFRs in the activities of the major CYP isoforms and phase 2 enzymes, UGT and SULT, CYP inductions, and transporters in PHHs (Tables 2-4). These values should be the "benchmarks" to evaluate the hepatic functions of newly developed hiPSC-derived hepatocytes as alternatives to PHHs in pharmaceutical ADMET studies. We recommend that the researchers studying hiPSC-derived hepatocytes select PHHs as controls referring the MFRs and provide the data on these evaluation points by similar methods to identify the characteristics of the newly developed hiPSC-derived hepatocytes. Such data are very helpful not only for pharmaceutical ADMET researchers, but also for hiPSC-derived hepatocytes researchers or vendors to evaluate the hepatic functions of hiPSC-derived hepatocytes.

Our consortium, CSAHi showed that the activities of drug metabolizing enzymes, CYP inductions, and transporters in hiPSC-derived hepatocytes, which were commercially available in 2013, were much lower than the MFRs of PHHs identified in this study (data not shown). Moreover, our microarray analyses indicated that the gene
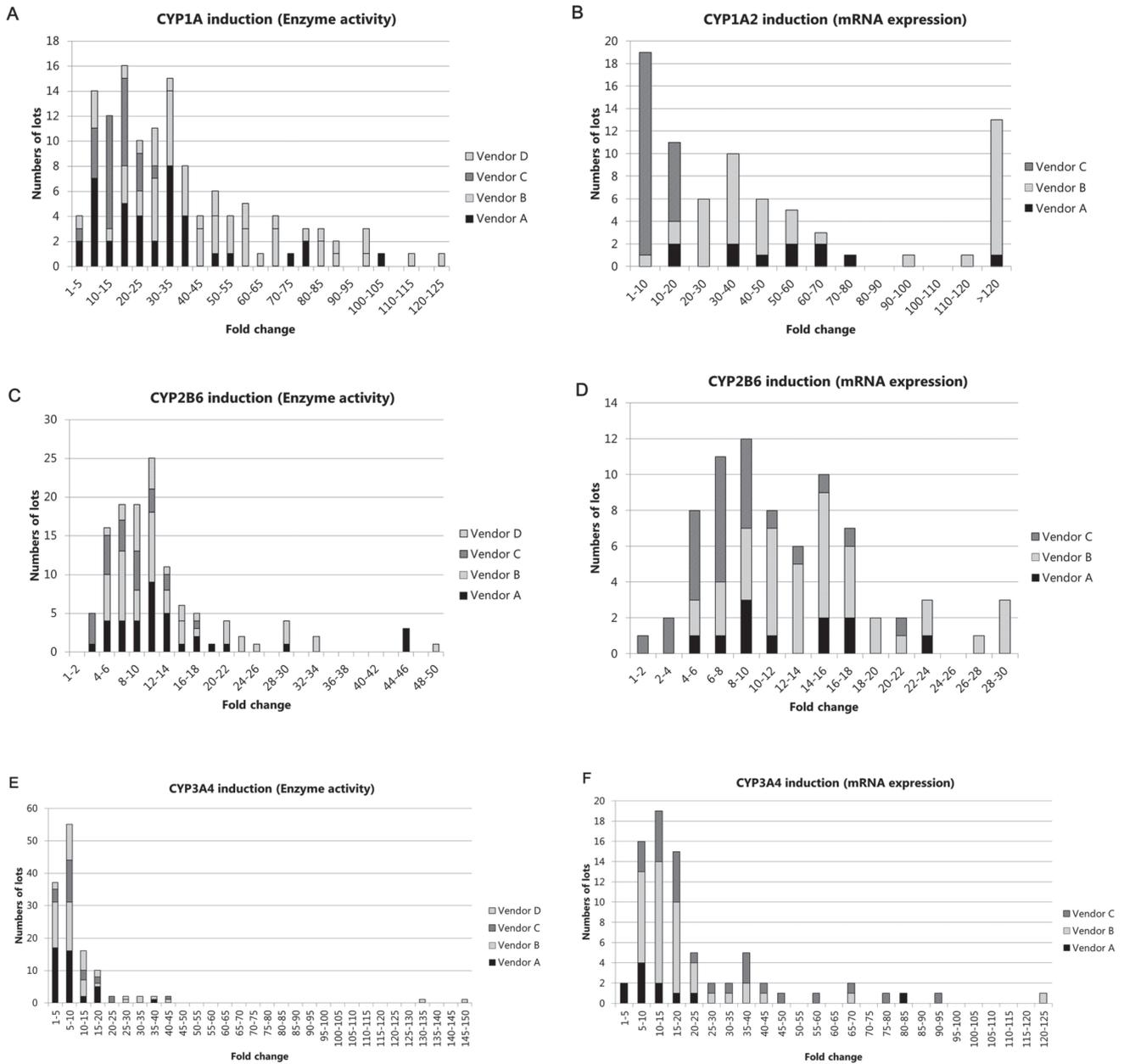
Fig. 4. Distributions of CYP induction activities in PHH. CYP induction activity of each enzyme in plated hepatocyte and numbers of lots was shown in the levels of enzyme activities and mRNAs. CYP1A2 induction by omeprazole was examined and its basal and induced activities were measured by phenacetin O-dealkylation. CYP2B6 induction by phenobarbital was examined and its basal and induced activities were measured by bupropion hydroxylation. CYP3A induction by rifampicin was examined and its basal and induced activities were measured by testosterone 6β-hydroxylation or midazolam 1'-hydroxylation.

expression profiles of hiPS-derived hepatocytes commercially available in 2013 were quite different to those of PHHs (Saito *et al*., in preparation). These results strongly suggested that these hiPSC-derived hepatocytes did not have sufficient hepatic function to be used as alternatives of PHHs. Some vendors recently have launched new versions of hiPSC-derived hepatocytes, which show improved drug metabolizing activities. Their potential as
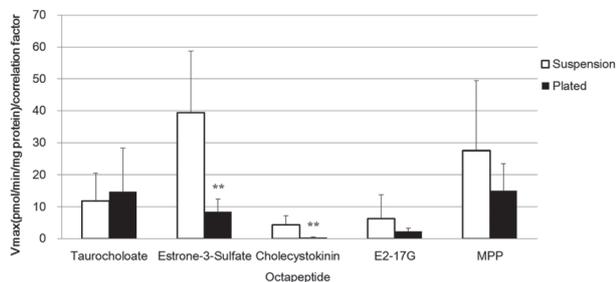
Fig. 5. Comparison of transporter activities between hepatocyte suspension and plated hepatocytes. Transporter activity of each isoform was compared to PHHs which were cultured in suspension (open) and plated (solid). Data shown are the mean + SD. **, p < 0.01.

alternatives of PHHs should be widely evaluated by pharmaceutical ADMET researchers.

To develop hiPSC-derived hepatocytes which have similar hepatic functions to PHHs, not only differentiation methods, but also the culture systems should be improved. There are many reports on the improvements of hepatic functions in hES/iPSC-derived hepatocytes by three-dimensional culture systems or spheroids (Gieseck *et al.*, 2014; Nagamoto *et al.*, 2012; Takayama *et al.*, 2013; Sengupta *et al.*, 2014). Further studies are needed to find the best combination of these culture methods and cell types and improve the functions of hiPSC-derived hepatocytes. Recent studies reported that the micro-patterned co-culture methods enhanced the hepatic functions of hiPSC-derived hepatocytes and could be useful to screen for DILI (Berger *et al.*, 2014; Ware *et al.*, 2015). They seeded hiPSC-derived hepatocytes on the micro-patterned collagen in a microwell plate, and co-cultured them with murine fibroblasts. They showed that the hiPSC-derived hepatocytes maintain their hepatic functions such as albumin production, urea production, and drug metabolizing activities for about one month (Berger *et al.*, 2014).

Another culture method to maturate hiPSC-derived hepatocytes is "*in vivo* maturation". Takebe *et al.* (2013)

succeeded in the formation of the liver like structure in nude mice by transplantation of the hiPSC-derived organ bud. Hepatocytes after *in vivo* maturation could be recollected and be used for the *in vitro* ADMET studies as more functional hepatocyte models to predict the risk of clinical DILI. Immunodeficient chimeric mice with human hepatocytes have been widely used in pharmaceutical ADMET research (Kakuni *et al.*, 2012; Schulz-Utermoehl *et al.*, 2012). The transplantation study of hiPSC-derived hepatocytes to immunodeficient mice has been reported very recently, but their drug metabolizing activities remained to be determined (Song *et al.*, 2015).

As mentioned above, there are large individual differences in the drug metabolizing activities, derived from gene polymorphism (Kurose *et al.*, 2012; Pressiner *et al.*, 2013; Zanger and Schwab, 2013). We need to evaluate many PHH lots to evaluate the risk of idiosyncratic DILI. However, due to the limitation of PHH supply, it has been quite difficult to evaluate the risk of idiosyncratic DILI sufficiently in non-clinical research. Takayama *et al.* (2014) reported that they produced hiPSCs from multiple PHHs derived from different donors and differentiated such hiPSCs into the hepatocytes by the same method. HiPSC-derived hepatocytes derived from donors who are poor metabolizers of CYP2D6 showed less CYP2D6 activity than hiPSC-derived hepatocytes derived from those having wild-type. They also showed that hiPSC-derived hepatocytes derived from CYP2D6 poor metabolizers were more sensitive to desipramine, which was detoxified by CYP2D6, than wild type hiPSC-derived hepatocytes. These results suggested that individual differences in the sensitivity to DILI could be evaluated by hiPSC-derived hepatocytes. Once hiPSCs are established from donors with rare SNPs, researchers are able to use these cells repeatedly for pharmaceutical research. HiPSC-derived hepatocytes of various donors produced by the same protocol are expected to have a similar quality of differentiation. Cell panels of hiPSC-derived hepatocytes from different donors might enable prediction of individual differences of drug metabolism and DILI risk

Table 4. MFR for drug transport activities for each transporter.

Transporter	Substrate	MFR (pmol/min/mg protein) /correlation factor
NTCP	Taurocholoate	5-10
OATP	Estrone-3-Sulfate	30-40
OATP1B3	Cholecystokinin Octapeptide	0-1
OATP1B1/3	E2-17G	2-3
OCT1	MPP	10-20

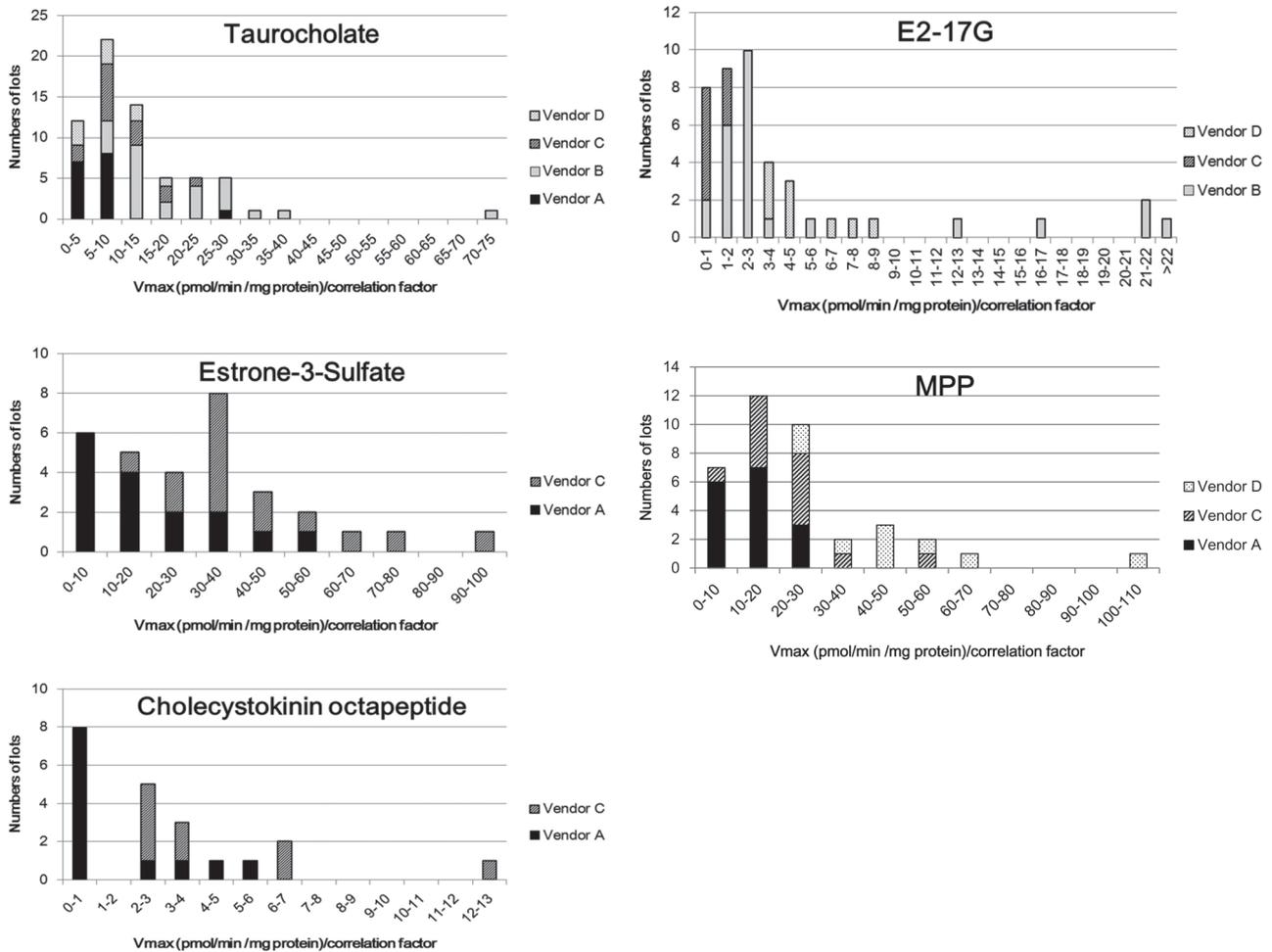MFR: most frequently observed value range.

Fig. 6. Distribution of efflux transporter activity. Efflux transporter activity was measured using the 5 compounds indicated as substrates.

in the human population precisely by non-clinical studies in the near future.

The MFRs that we showed here are not a goal, but those of benchmarks, for the functions of hiPSC-derived hepatocytes. Cryopreserved PHHs, the current gold standard, are not sufficient for clinical prediction because the activities of drug metabolizing enzymes in the cryopreserved PHHs decline immediately after thawing and seeding (Gebhardt *et al.*, 2003). Therefore, we should aim towards hiPSC-derived hepatocytes becoming alternatives to human liver, not PHHs, for more sophisticated pharmaceutical research in the future. We expect further development of hiPSC-derived hepatocytes which contribute to ensuring the safety of patients, reduction and replacement of animal studies, and acceleration of pharmaceutical research.

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