Supplemental Information

Validation of uPA/SCID mouse with humanized liver as a human liver model: protein quantification of transporters, cytochromes P450, and UDP-glucuronosyltransferases by LC-MS/MS

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Drug Metabolism and Disposition

Supplemental Table 1 Chimeric mice used in the present study.

Number	Human hepatocyte donor	Days after transplantation	Sex of chimeric mouse	Replacement index (%)
1-1	BD85	68	Female	76.8
1-2	BD85	68	Female	87.5
1-3	BD85	68	Female	86.7
1-4	BD85	68	Female	79.7
1-5	BD85	68	Female	90.0
2-1	BD87	68	Male	98.6
2-2	BD87	68	Male	96.8
2-3	BD87	75	Male	96.9
3-1	BD72	69	Female	71.0
3-2	BD72	75	Male	74.4
3-3	BD72	75	Male	96.6

Cryopreserved human hepatocytes from donors BD85 (black, male, 5 years old), BD72 (white, female, 10 years old) and BD87 (white, male, 2 years old) were transplanted into urokinase-type plasminogen activator/severe combined immunodeficiency mice to generate chimeric mice. The replacement index, which is the rate of the replacement from mice to human hepatocytes, was measured using anti-human specific cytokeratin 8 and 18 antibody (Tateno et al., 2004).

Supplemental Table 2 Amino acid sequences of target peptides used for quantification, and detection of these peptides in mouse liver microsomal fraction and plasma membrane fraction.

Protein	Peptide sequence	Mouse liver	
		Microsomal fraction	Plasma membrane fraction
CYP2E1	GIIFNNGPTWK [a]	Detected	
CYP3A4	LQEEIDAVLPNK	N.D.	
CYP2C9	GIFPLAER	N.D.	
CYP1A2	YLPNPALQR	N.D.	
CYP2C8	GNSPISQR	N.D.	
CYP2A6	GTGGANIDPTFFLSR	N.D.	
CYP2C19	GHFPLAER	N.D.	
CYP2D6	DIEVQGFR	N.D.	
CYP3A7	FNPLDPFVLSIK	N.D.	
CYP3A5	DTINFLSK	N.D.	
CYP4A11	IPIPIAR	N.D.	
CYP2B6	GYGVIFANGNR	N.D.	
CYP3A43	YIPFGAGPR	N.D.	
P450R	FAVFGLGNK [a]	Detected	
UGT2B7	TILDELIQR	N.D.	
UGT1A1	TYPVPFQR	N.D.	
Na^+/K^+ ATPase	AAVPDAVGK [a]	Detected	Detected
γ-GTP	LFQPSIQLAR [a]	N.D.	N.D.
MDR3	STTVQLIQR		N.D.
BSEP	NLVFAQR		N.D.
MDR1	FYDPLAGK		N.D.
MRP2	QLLNNILR		N.D.
MATE1	GGPEATLEVR		N.D.
ABCG8	ASLLDVITGR [a]		Detected
BCRP	VSSYFLGK		N.D.
OCT1	LSPSFADLFR		N.D.
OAT2	NVALLALPR		N.D.
NTCP	GIYDGDLK [a]		Detected
MRP6	APETEPFLR		N.D.
OATP1B1	LNTVGIAK		N.D.
OATP1B3	IYNSVFFGR		N.D.
OATP2B1	VLLQTLR		N.D.
ENT1	WLPSLVLAR		N.D.

The specificity of amino acid sequences was checked against the UniProt database of human and mouse proteins. Each target peptide is unique for the human target, except for the peptides of CYP2E1, P450R, Na^+/K^+ ATPase, γ-GTP, ABCG8 and NTCP. Trypsin-digested microsomal or plasma membrane fraction of ddY mouse liver was analyzed by LC-MS/MS

(API5000, AB Sciex) after spiking with stable isotope-labeled internal standard peptides. The target peptide was considered detected when the peak was detected in 3 or 4 MRM transitions at the same retention time as that of the internal standard peptide.

[a] Amino acid sequence of tryptic peptide is conserved in both human and mouse proteins.

N.D., not detected (no peak was detected, or only one or two peaks were detected, at the same retention time as that of the internal standard peptide).

The quantified peptides of 6 proteins (CYP2E1, P450R, Na^+/K^+ ATPase, γ-GTP, ABCG8 and NTCP) are conserved in human and mouse, and therefore quantified proteins of both species in liver. Since mouse γ-GTP was not detected in microsomal or plasma membrane fraction of mouse liver (Supplemental table 2), the quantified values in those fractions of PXB mouse liver were considered to represent human γ-GTP. However, the quantified values of the other 5 proteins in PXB mouse represent the sum of human protein and the mouse protein in remaining mouse hepatocytes. The average remaining percentage of mouse hepatocytes according to donor was estimated to be 15.9 % for BD85, 2.57 % for BD72 and 19.3 % for BD87, calculated as 100 – RI (%), as shown in Supplemental table 1. Although the greatest difference was 7.5-fold between mice derived from BD72 and BD87, such differences were not observed in the quantified values of these 5 proteins in Tables 1 and 2, suggesting that the quantified values represented mainly human proteins expressed in replaced human hepatocytes.

Supplemental Methods

Preparation of microsomal and plasma membrane fractions

Mice were euthanized by cardiac puncture under anesthesia with ether. The livers were isolated and perfused with saline to remove blood, and then frozen in liquid nitrogen immediately. Frozen liver tissues were dissected and homogenized using a Potter-Elvehjem homogenizer in buffer A (0.1 M KCl-phosphate buffer (pH 7.4) containing a protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO). The obtained homogenates were centrifuged at 10,800 × g for 20 min at 4°C and the supernatants were collected and ultracentrifuged at 100,000 × g for 60 min at 4°C. The microsomal pellet was suspended in buffer A and ultracentrifuged at 100,000 × g for 60 min at 4°C. The resulting pellet was suspended in buffer B (20 mM Tris-HCl-buffer containing 0.25 M saccharose and 5.4 mM EDTA), and part of the solution was stored at -80 °C as microsomal fraction (Kawakami et al., 2011). The remaining portion was layered on top of a 38% (w/v) sucrose solution and centrifuged at 100,000 x g for 30 min at 4°C. The turbid layer at the interface was recovered, suspended in buffer B, and centrifuged at 100,000 x g for 30 min at 4°C. The plasma membrane fraction was obtained from the resulting pellet, which was suspended in buffer B. Protein concentrations were measured by the Lowry method using the DC protein assay reagent (Bio-Rad, Hercules, CA).

LC-MS/MS-based protein quantification analysis

Absolute amounts of each target peptide were determined by using an internal standard peptide, which is a stable isotope-labeled peptide with the identical amino acid sequence to that of the corresponding target peptide. The amino acid sequences of target peptides are shown in Supplemental table 2. The sequence specificity of each peptide was checked against the UniProt database of human and mouse proteins. All peptides, except CYP2E1, P450R, Na^+/K^+ ATPase, γ-GTP, ABCG8 and NTCP, were specific for human target proteins, and were not detected in

microsomal or plasma membrane fraction of mouse liver. The peptides of CYP2E1, P450R, Na^+/K^+ ATPase, γ-GTP, ABCG8 and NTCP were specific for both human and mouse target proteins, and were detected in either fraction of mouse liver, except for γ-GTP, as shown in Supplemental table 2. The peptide of γ-GTP was not detected in either microsomal or plasma membrane fraction. The detailed MRM conditions for the peptides were reported previously (Kamiie et al., 2008; Ohtsuki et al., 2011; Sakamoto et al., 2011; Shawahna et al., 2011; Uchida et al., 2011).

Protein samples were suspended in suspension buffer containing 7 M guanidium hydrochloride and 10 mM EDTA. Samples were reduced with dithiothreitol at room temperature for 60 min under a nitrogen atmosphere, and S-carbamoylmethylated by treatment with iodoacetamide at room temperature for 60 min. The alkylated proteins were precipitated with a mixture of methanol and chloroform. The precipitates were dissolved in 6 M urea, diluted with 100 mM Tris-HCl (pH 8.0) and digested with tosylphenylalanyl chloromethyl ketone-treated trypsin at an enzyme/substrate ratio of 1:100 at 37°C for 16 hours.

The tryptic digests were spiked with stable isotope-labeled internal standard peptides and acidified with formic acid for analysis with the nano LC system or HPLC system, which was connected to an electrospray ionization triple quadrupole mass spectrometer (QTrap5500 for transporters or API5000 for metabolizing enzymes; AB Sciex, Foster City, CA, USA), operated in the positive ionization mode. LC was performed with C18 columns; L-column 0.1 mm ID x 5 mm (Chemical Evaluation and Research Institute, Tokyo, Japan) for nanoLC and ZORBAX SB-C18 1.0 mm ID x 150 mm (Agilent, Santa Clara CA). Linear gradients of 1-45% acetonitrile in 0.1% formic acid were applied to elute the peptides at a flow rate of 200 nl/min for nano LC (DIONEX Ultimate 3000, DIONEX, Sunnyvale, CA) or 50 μl/min for conventional LC (Agilent 1100, Agilent). The mass spectrometer was set up to run a multiplexed-MRM experiment for peptides. The ion counts in the chromatograms were determined by using the

quantitation procedures in Analyst software (AB Sciex).

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