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In vitro Screening System for Hepatotoxicity: Comparison of Bone-Marrow-Derived Mesenchymal Stem Cells and Placenta-Derived Stem Cells

Hyun-Jung Lee,[1] Kyeung Eun Cha,[2] Seong-Gyu Hwang,[3] Jin Kyeoung Kim,[4] and Gi Jin Kim[2*]

[1] *Cha Bio and Diostech Co., Ltd, CHA Stem Cell Institute, Seoul 135-081, Republic of Korea*
[2] *Department of Biomedical Science, CHA Stem Cell Institute, CHA University, Seoul 135-097, Republic of Korea*
[3] *Department of Internal Medicine, Bundang CHA Hospital, CHA University, Gyeonggi-do 463-712, Republic of Korea*
[4] *Department of Biomedical Science, CHA University, Bundang-gu 463-836, Republic of Korea*

ABSTRACT

Stem cells have unique properties such as self-renewal, plasticity to generate various cell types, and availability of cells of human origin. The characteristics are attentive in the toxicity screening against chemical toxicants. Placenta-derived stem cells (PDSCs) have been spotlighted as a new cell source in stem cell research recently because they are characterized by their capacity to differentiate into multilineages. However, the use of PDSCs as an in vitro screening model for potential drug candidates has not yet been studied. Here, we analyzed the potentials for bone-marrow-derived mesenchymal stem (BM-MSCs), which is a representative adult stem cells and PDSCs as an in vitro hepatotoxicity screening system, using well-known hepatotoxicants. BM-MSCs and PDSCs were analyzed to the potential for hepatogenic differentiation and were cultured with different concentrations of hepatotoxicants for time courses. The viability and ATP-binding cassette (ABC) transporters were measured by the MTT assay and RT-PCR, respectively. The sensitivities of PDSCs to hepatotoxicants are more sensitive than those of BM-MSCs. The viability (IC_{50}) to in PDSCs was less than that of BM-MSCs after 48 and 72 h ($P < 0.05$) of CCl_4 exposure. The toxicities of CCl_4 were decreased by fourfold in hepatogenic differentiation inducing PDSCs compared to the undifferentiated cells. The alteration of ABCGs was observed in PDSCs during differentiation. These findings suggest that the naïve PDSCs expressing ABCGs can be used as a source for in vitro screening system as well as the expression patterns of ABCG1 and ABCG2 might be involved in the sensitivity of PDSCs to hepatotoxicants. J. Cell. Biochem. 112: 49–58, 2011.

KEY WORDS: PLACENTA-DERIVED STEM CELLS; BONE-MARROW-DERIVED MESENCHYMAL STEM CELLS; IN VITRO SCREENING SYSTEM; HEPATOTOXICITY; ATP-BINDING CASSETTE (ABC) TRANSPORTERS

Toxicological screening using animals are necessary for drug development registration. Because this approach is time-consuming, costly, labor intensive, stressful for the animals, and susceptible to inaccuracies due to individual differences between animals, the screening of candidate chemicals in early development is often replaced with in vitro cell culture systems [Pearson, 1986; Liebsch and Spielmann, 2002]. In vitro studies using cell lines were capable, or potentially capable, of providing more rapid, precise, relevant information than some animal studies, and economical approach for the evaluation of the pharmaco-toxicological profiling of target drugs, characterized by a low compound requirement and short duration [Pearson, 1986; Kari et al., 2007]. Also, it is possible to include mechanistic studies, and to test for toxicity that is specific to humans: sensitivity differences between humans and rodents can affect animals [Kari et al., 2007]. Among the in vitro screening systems, primary cell cultures and/or target organ-specific cell lines can be used to measure the general toxicity of a test compound [Zhou et al., 2006].

However, the sensitivity of hepatotoxicity using primary human hepatocytes or the HepG2 cell line cannot predict effects in early development and toxicological differences, which depend on the state of differentiation in hepatocytes [Knasmuller et al., 2004; Xu

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*Correspondence to: Prof. Gi Jin Kim, Department of Biomedical Science, CHA University, 606-16 Yeoksam-1dong, Kangnam-ku, Seoul 135-097, Republic of Korea. E-mail: gjkim@cha.ac.kr

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et al., 2004]. In addition, primary cells such as hepatocytes in particular and many transformed human hepatocyte-derived cell lines (immortalized cultures, i.e., Fa2-N4 cells, HepaRG cells) were developed have limitations in their life span and can have donor-dependent variations [Mills et al., 2004]. However, they have disadvantages such as discontinuous phenotypic characteristics, functional properties, and genetic instability. Therefore, more promised future is waiting for hepatocyte-like cells as the source of hepatocytes regarding the approach of stem cells use in the high-throughput testing [Duret et al., 2007; Ishii et al., 2008].

Stem cells have many advantages over primary cells and immortalized cell lines for in vitro toxicity screening, including unique properties such as unlimited self-renewal, plasticity to generate various cell types, and availability of cells of human origin [Davila et al., 2004; Kulkarni and Khanna, 2006; Zhang and Wang, 2008]. In vitro assaying of embryotoxicity using embryonic stem cells for the early determination of the teratogenic potential of a compound has been attempted [Kim et al., 2006; Kulkarni and Khanna, 2006]. However, sensitivity to cytotoxic chemicals in embryonic stem cells has been shown to depend on methods or efficiencies of differentiation [Seiler et al., 2004; Kim et al., 2008; Kim et al., 2009]. Especially the expression of ABCG2 multidrug transporter, which is one of the important factors in ABC transporters (ATP-binding cassette (ABC) transporters) highly increased in undifferentiated human embryonic stem cells [Apáti et al., 2008]. The expression of ABCG2 regulates the role of several toxicants to cells via ABCG2 function and its variants in cancer therapy and toxicology [Sarkadi et al., 2004; Cervenak et al., 2006]. These results suggest that cytotoxicity sensitivity may differ according to the developmental stage of the embryonic stem cells. Nevertheless, guidelines for defined differentiation toward target cells have not been established. Stable and sensitive in vitro screening systems have been required regardless of discrepancy in differentiation. Therefore, in vitro screening system using adult stem cells was introduced [Albella et al., 2002; Shuga et al., 2007]. Bone-marrow-derived mesenchymal stem cells (BM-MSCs) are one of the representative adult stem cells. It has a differentiation potential into multilineages including osteogenic, chondrogenic, adipogenic, neurogenic, and hepatogenic lineages [Kassem and Abdallah, 2008].

Recently, it was reported that placenta-derived stem cells (PDSCs) that were isolated from a full-term placenta were capable of adhering to plastic ware, forming fibroblast colony-forming units, expressing a pattern of stem cell-specific surface antigens, and showing a differentiation potential toward one or more lineages that included osteogenic, adipogenic, chondrogenic, and hepatogenic types [Fukuchi et al., 2004; Yen et al., 2005; Chien et al., 2006; Parolini et al., 2008]. Furthermore, PDSCs, which are organ-specific cells derived from a fetus, are a primitive cell type and an intermediate between embryonic and adult stem cells [In 't Anker et al., 2004].

The objectives of the present study were to compare BM-MSCs and PDSCs in the potential for hepatogenic differentiation and explore in the sensitivity of them against hepatotoxicants including *N*-nitrosodiethylamine (DEN), *N*-nitrosodimethylamine (DMN), carbon tetrachloride (CCl_4), well-known hepatotoxicants and widely used agents for animal hepatotoxicity models, analyze the expression and their correlation of ABCGs in between BM-MSCs and PDSCs depending on differentiation, and to determine if PDSCs could use a source for hepatotoxicity screening.

MATERIALS AND METHODS

CULTURING OF PDSCs AND BM-MSCs

Human normal placentas, which had no evidence of medical, obstetrical, or surgical complications, were collected after term delivery (≥37 gestational ages). All women provided written, informed consent prior to the collection of samples. The collection of samples and their utilization for research purposes were approved by the Institutional Review Board of CHA General Hospital, Seoul, Korea. To isolate PDSCs, the chorioamniotic membrane was peeled off and separated from the chorionic plate of placenta. They were chopped into small pieces, then washed with phosphate-buffered saline (PBS), and initially digested with 0.5% collagenase IV in PBS at 37°C for 40 min, followed by vigorous shaking for 15 min at 15-min intervals over 90 min in 0.25% collagenase IV at 37°C. An equal volume of Dulbecco's modified Eagle's medium (DMEM; Gibco-Invitrogen, Grand Island, NY) containing 10% fetal bovine serum (FBS; Gibco-Invitrogen) was added and centrifuged at 1,000*g* for 5 min to get the cell pellet. The harvested cells were resuspended in the culture medium and then incubated at 37°C in an incubator with 5% CO_2.

BM-MSCs (Cambrex Bioscience Walkersville, East Rutherford, NJ) were cultured using a culture medium containing alpha-MEM (Gibco-BRL-Invitrogen), supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 1 mM sodium pyruvate (Gibco-BRL), and 10% FBS (Gibco-BRL). Isolated PDSCs were cultured with culture medium contained DMEM/F12 (Gibco-BRL) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 25 ng/ml FGF4 (Peprotech, Inc., NJ), 1 μg/ml heparin (Sigma), 50 μg/ml gentamicin (Gibco-BRL), and 10% FBS (Gibco-BRL).

REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

For RT-PCR analysis, BM-MSCs and PDSCs were homogenized and lysed in 1 ml of TRIzol (Invitrogen, Carlsbad, CA). Total RNA was extracted with 200 μl of chloroform and precipitated with 500 μl of 80% (v/v) isopropanol. After the supernatant was removed, the RNA pellet was washed with 75% (v/v) ethanol, air-dried, and dissolved in 0.1% (v/v) diethyl pyrocarbonate-treated water. The RNA concentration was determined by measuring absorbance at 260 nm using a spectrophotometer. A reverse transcription reaction was performed with 1 μg of total RNA and SuperScript™ III reverse transcriptase (Invitrogen). The cDNA was amplified using h-Taq DNA polymerase (Solgent, Seoul, Korea), according to the manufacturer's instructions. First-strand cDNAs were amplified in a final volume of 20 μl containing 0.5 U Taq DNA polymerase (Solgent) and 20 pmol of each human-specific target primers. The PCR primers and the size of the amplified products are shown in Table I. Amplification reactions were performed on the following conditions: denaturation at 95°C for 15 min followed by 35–40 cycles of denaturation at 95°C for 30 s, annealing at 50–60°C for 40 s, and elongation at 72°C for 5 min. The PCR products were visualized and photographed following electrophoresis on a 1% (w/v) agarose gel containing 0.5 μg/ml ethidium

TABLE I. Primers Used for Reverse Transcription Polymerase Chain Reaction

Genes	Sequences	T_m (°C)	Size (bp)
OCT4	F: 5′-ACA CTC GGA CCA CGT CTT TC-3′ R: 5′-CGT TCT CTT TGG AAA GGT GTT C-3′	54	300
Nanog	F: 5′-TTC TTG ACT GGG ACC TTG TC-3′ R: 5′-GCT TGC CTT GCT TTG AAG CA-3′	54	300
Sox2	F: 5′-GGG CAG CGT GTA CTT ATC CT-3′ R: 5′-AGA ACC CCA AGA TGC ACA AC-3′	52	200
NF-68	F: 5′-GAG TGA AAT GGC ACG ATA CCT A-3′ R: 5′-TTT CCT CTC CTT CTT CTT CAC CTT C-3′	58	500
Cardiac	F: 5′-GGA GTT ATG GTG GGT ATG GGT C-3′ R: 5′-AGT GGT GAC AAA GGA GTA GCC A-3′	58	500
hAFP	F: 5′-AGC TTG GTG GAT GAA AC-3′ R: 5′-TCC AAC AGG CCT GAG AAA TC-3′	50	200
HLA-G	F: 5′-GCG GCT ACT ACA ACC AGA GC-3′ R: 5′-GCA CAT GGC ACG TGT ATC TC-3′	58	550
TERT	F: 5′-GAG CTG ACG TGG AAG ATG AG-3′ R: 5′-CTT CAA GTG CTG TCT GAT TCC AAT G-3′	55	300
Albumin	F: 5′-CCC CAA GTG TCA ACT CCA AC-3′ R: 5′-CTC CTT ATC GTC AGC CTT GC-3′	54	450
TAT	F: 5′-AAC GAT GTG GAG TTC ACG G-3′ R: 5′-GAC ACA TCC TCA GGA GAA TGG-3′	59	288
ABCG1	F: 5′-TTT GAG GGA TTT GGG TCT GAA C-3′ R: 5′-CCC CTT TAA TCG TTT TGT CTG CT-3′	58	229
ABCG2	F: 5′-AAC GAT GTG GAG TTC ACG G-3′ R: 5′-CAC ACT CTG ACC TGC TGC TAT G-3′	62	312
ABCA1	F: 5′-AAC TCT ACA TCT CCC TTC CCG-3′ R: 5′-CTC CTG TCG CAT GTC ACT CC-3′	58	123
ABCA2	F: 5′-AGT GCT CAG CCT TCG TAC AG-3′ R: 5′-AGG CGC GTA CAG GAT TTT GG-3′	56	188
ABCA3	F: 5′-ACG GTC CTG GAA CTC TTC CT-3′ R: 5′-TCT GAG AAG GGA TGT AGG CAA-3′	56	187
β-Actin	F: 5′-TCC TTC TGC ATC CTG TCA GCA-3′ R: 5′-CAG GAG ATG GCC ACT GCC GCA-3′	58	300

bromide. cDNA samples were adjusted to yield equal actin amplifications.

FACS ANALYSIS

For FACS analysis, PDSCs (5×10^5 cells) were dissociated with cell dissociation buffer (Gibco-Invitrogen) and washed with PBS containing 2% (v/v) FBS. They were incubated with isotype control IgG or antigen-specific antibodies (BD Biosciences, San Diego) for 20 min and propidium iodide (PI, 5 ng/ml; Sigma) was used to identify nonviable cells. FACS sorting was performed using a FACS vantage Flow Cytometer (BD Biosciences, San Jose, CA).

HEPATOGENIC DIFFERENTIATION

To induce the differentiation of BM-MSCs and PDSCs into hepatocytes in vitro, we designed a series of induction media, listed in Table II. As an initiation step, the cells were cultured on 0.1% collagen-coated dishes in culture medium containing 2% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. When these cells were grown to 70–80% confluence, about 2 days later, the culture medium was replaced with early induction medium to allow for hepatic differentiation. The induction protocol was designed in three steps, with different combinations of epithelial growth factor (EGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), and bone morphogenetic proteins 4 (BMP4) as indicated in Table I. After the terminal step 7 days later, the inducing cells were analyzed using indocyanine green (ICG, Dongindang Pharm. Co., Ltd) for the identification of hepatic function. After that, the hepatic inducing cells were harvested for the analysis of hepatocyte-specific genes expression using RT-PCR analysis.

INDOCYANINE GREEN (ICG) UPTAKE ASSAY

After the final hepatogenic differentiation steps, the cells were washed with PBS, added to the plates at a final concentration of 1 mg/ml ICG, and incubated at 37°C for 1 h 30 min. The cells were then rinsed three times with PBS and the dishes were refilled with DMEM containing 10% FBS. After that, uptake of ICG in BM-MSCs and PDSCs was measured using an inverted microscope. ICG was completely eliminated from the cells 72 h later in the ICG uptake cells.

UREA ASSAY

Undifferentiated and differentiated BM-MSC and PDSC were cultured for 24 h in media supplemented. Supernatants were collected and centrifuged. Urea concentrations were determined using a colorimetric assay (Quantichrom Urea Assay Kit, Bioassay Systems). Fresh culture media were used as negative controls.

CYTOTOXICITY ASSAY

The DEN (Sigma) and DMN (Nacalai Tesque, Japan) chemicals were diluted in saline and the CCl_4 (Daejung, Co., Ltd, Korea) was diluted in DMSO. The toxicity dosages used for hepatotoxicants are modified with Sauer et al. [1995] and Jeong et al. [1994]. The hepatotoxin cytotoxicity was assayed in 96-multiwell culture plates. BM-MSCs and PDSCs were seeded at a concentration of 4×10^3 cells/well. At 80% confluence, these cells were treated with various concentrations of chemicals for 24, 48, and 72 h. Cytotoxicity was estimated using the MTT assay, according to the protocol described by the manufacturer. Briefly, 10 μl of MTT (5 mg/ml in PBS, Sigma) was added to each well and incubated for 4 h. The media were discarded and 50 μl of dimethyl sulfoxide (DMSO) was added. The plate was gently mixed on a gyratory shaker at room temperature for 30 min. Optical density was measured at 562 nm. Cytotoxicity was defined as the concentration that reduced cell viability to 50% (IC_{50}), compared to the control level. From this, the IC_{50} concentrations of the test compounds including DEN, DMM, and CCl_4 were selected.

TABLE II. Composition of Hepatogenic Differentiation Inducing Medium

Inducing medium	Incubation	Growth factors
Culture medium	1–2 days	None
Early inducing medium	2 days	EGF (20 ng/ml), b-FGF4 (10 ng/ml), BMP-4 (10 ng/ml)
Mid-term inducing medium	7 days	b-FGF4 (10 ng/ml), HGF (20 ng/ml)
Terminal inducing medium	7 days	Oncostatin M (20 ng/ml), DEXA (1 μM), 1× insulin transferrin selenium

WESTERN BLOT ANALYSIS

The lysates from cells were sonicated for 30 s with protein extraction solution (Intron, Kyunggi, South Korea) and centrifuged at 12,000 rpm for 15 min at 4°C. Total protein concentrations of the supernatants were determined with a BCA protein assay kit (Pierce, Rockford, IL). Total protein extracts (40 μg) were heated at 95°C for 5 min, resolved by 12% SDS–polyacrylamide gel electrophoresis (PAGE), and electrotransferred onto PVDF membranes at 100 V for 80 min. The membranes were blocked for 1 h at room temperature in 5% nonfat milk powder in TBS-T (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Tween-20) and incubated with overnight at 4°C with primary antibodies to ABCG1 (Novus Biological), ABCG2 (Chemicon), or β-actin (Santa Cruz Biotechnology). The filters were then washed in TBS-T, followed by horseradish peroxidase (HRP)-conjugated rabbit or mouse secondary antibodies (Bio-Rad). Peroxidase activity was visualized with an ECL Advance Western blotting detection kit (Amersham, Piscataway, NJ).

STATISTICS

Results shown are the average of three independent experiments ($n = 3$). The values are expressed as means ± SD. Urea assay was analyzed by one-way ANOVA. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

The morphologies of PDSCs were similar to those of traditional mesenchymal stem cells (Fig. 1A). As shown in Figure 1B, expression of Oct-4, which is a specific marker of embryonic stem cells, was not detected in PDSCs, Nanog, and TERT genes, which are strongly proliferation markers, and three germ layer markers, SOX2, NF68, Cardiac, and AFP were expressed in PDSCs compared to CHA3—human embryonic stem cells (a NIH registered hESC). Also, HLA-G, which is involved in immunosuppressor property, was expressed. These results indicated that PDSCs have potential for self-renewal and differentiation into multiple lineages. To confirm the surface phenotypes of PDSCs, we performed FACS analysis using various mouse anti-human antibodies (Fig. 1C). The phenotypes of the PDSCs were similar to those of MSCs derived from adult BM-MSCs, that is, negative for hematopoietic markers such as CD34, CD45, and HLA-DR, and positive for nonhematopoietic markers including CD13, CD44, CD71, CD90, CD105, HLA-ABC, and HLA-G. There were no differences in the expression of any of these markers in PDSCs and BM-MSCs.

The morphologies of BM-MSCs and PDSCs were similar to those of traditional mesenchymal stem cells before inducing the hepatogenic differentiation (Fig. 2A, upper). To investigate the differentiation potential of BM-MSCs and PDSCs into

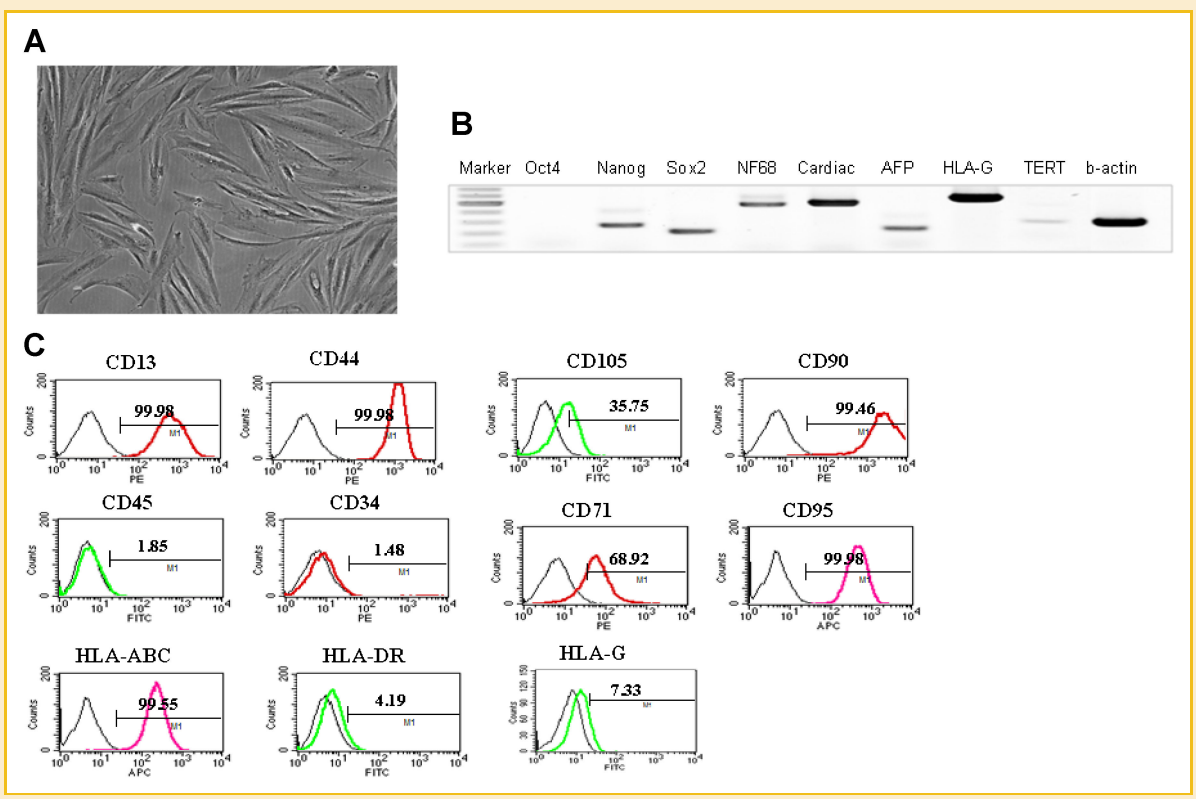

Fig. 1. Characterization of human placenta-derived stem cells (hPDSCs) isolated from human normal placentas. A: Morphology of hPDSCs isolated from human normal placentas is spindle shaped. B: RT-PCR to analyze the differentiation potential of hPDSCs into multilineages. C: Surface patterns of hPDSCs involved in immunosuppressive property. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

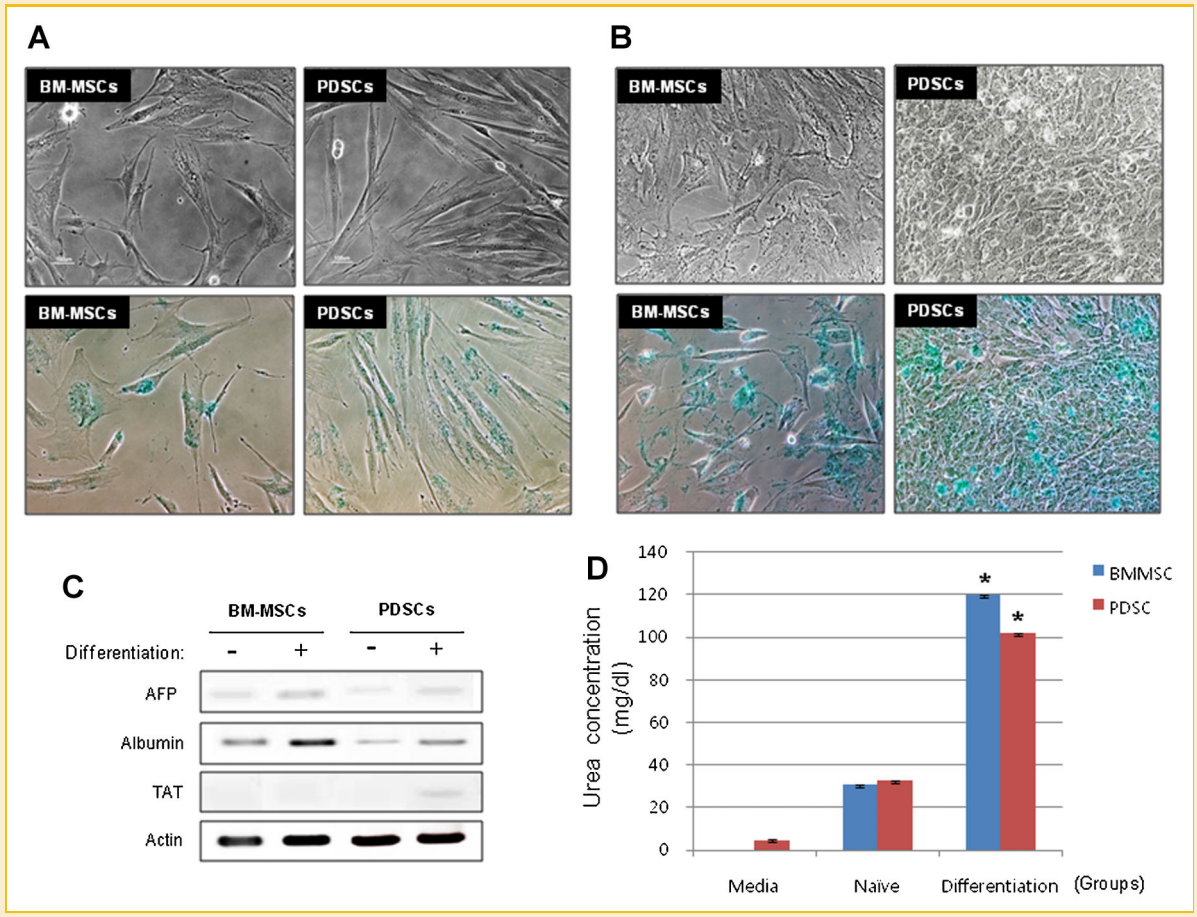

Fig. 2. Comparison of cell morphology and expression of hepatocyte-specific genes between bone marrow-mesenchymal stem cells (BM-MSCs) and placenta-derived stem cells (PDSCs) depending on hepatogenic differentiation. A: The morphology of BM-MSCs and PDSCs was similar to the round-spindle shape of mesenchymal stem cells (upper) and undifferentiated BM-MSCs and PDSCs exhibited a low level of ICG uptake (lower). B: The morphology of BM-MSCs and PDSCs changed to a round form, similar to that of hepatocytes, following hepatogenic differentiation (upper) and PDSCs exhibited more ICG uptake than BM-MSCs after hepatogenic differentiation (lower). C: Expression of hepatogenic-specific genes (AFP, albumin, and TAT) in BM-MSCs and PDSCs during hepatogenic differentiation by RT-PCR analysis. D: Urea production in BM-MSCs and PDSCs differentiated into hepatocyte-like cells was increased twofold compared to that in undifferentiated status. Original magnification: 100×. Data are expressed as means ± SD. $^{*}P < 0.05$ compared with naïve. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

hepatocytes, hepatogenic differentiation was induced in two separate steps including initiation step and maturation step. After hepatogenic differentiation, BM-MSCs and PDSCs gradually exhibited a morphological change from spindle shape to a polygonal outline, revealing characteristic hepatocyte-like morphology (Fig. 2B, upper). The frequencies of round-like structures were higher in PDSCs than BM-MSCs. Since these results are considered to represent a progressive gain of hepatic function, we performed the ICG uptake assay. ICG is a nontoxic organic anion clinically used to test hepatic function. Hepatocytes are known to uptake and release ICG in vivo [Schmelzer et al., 2007]. In the ICG uptake assay, hepatogenic differentiation-inducing PDSCs showed a more significant uptake of ICG than BM-MSCs (Fig. 2A,B, lower). The ICG that was uptaken by the cells was observed in round-like cellular structures whose morphology changed after hepatogenic differentiation. The expressions of hepatocyte-specific genes were examined using RT-PCR analysis (Fig. 2C). Hepatogenic differentiation inducing cells expressed more hepatocyte-specific genes such as *AFP* (alpha fetoprotein), albumin, and *TAT* (tyrosine amino transferase) than undifferentiated cells (naive cells). ELISA assay was used to analyze urea production in hepatogenic differentiation-inducing cells (Fig. 2D). Urea production was shown to increase twofold both in BM-MSCs and PDSCs after hepatogenic differentiation. These results demonstrate that BM-MSCs and PDSCs successfully differentiated into hepatocyte-like cells. Furthermore, hepatogenic differentiated hepatocyte-like cells function like hepatocytes, as confirmed by the ICG uptake and urea production assays.

To investigate the cytotoxicity of DEN, BM-MSCs and PDSCs were exposed to different doses (0–0.8 mM) of DEN for 24, 48, and 72 h (Fig. 3A). The concentration of DEN for 50% reduction (IC_{50}) in 24 h was 0.8 and 0.7 mM in BM-MSCs and PDSCs. In 48 h, the value of IC_{50} in BM-MSCs and PDSCs were 0.7 and 0.55 mM, respectively. The cytotoxicity of DEN in BM-MSCs was 0.6 mM, which was higher dosing that of PDSCs in 72 h. These results suggested that the sensitivity of PDSCs to DEN was higher than that of BM-MSCs.

In order to analyze the differences in cytotoxicity of DMN in BM-MSCs and PDSCs, the various cells were exposed to different doses

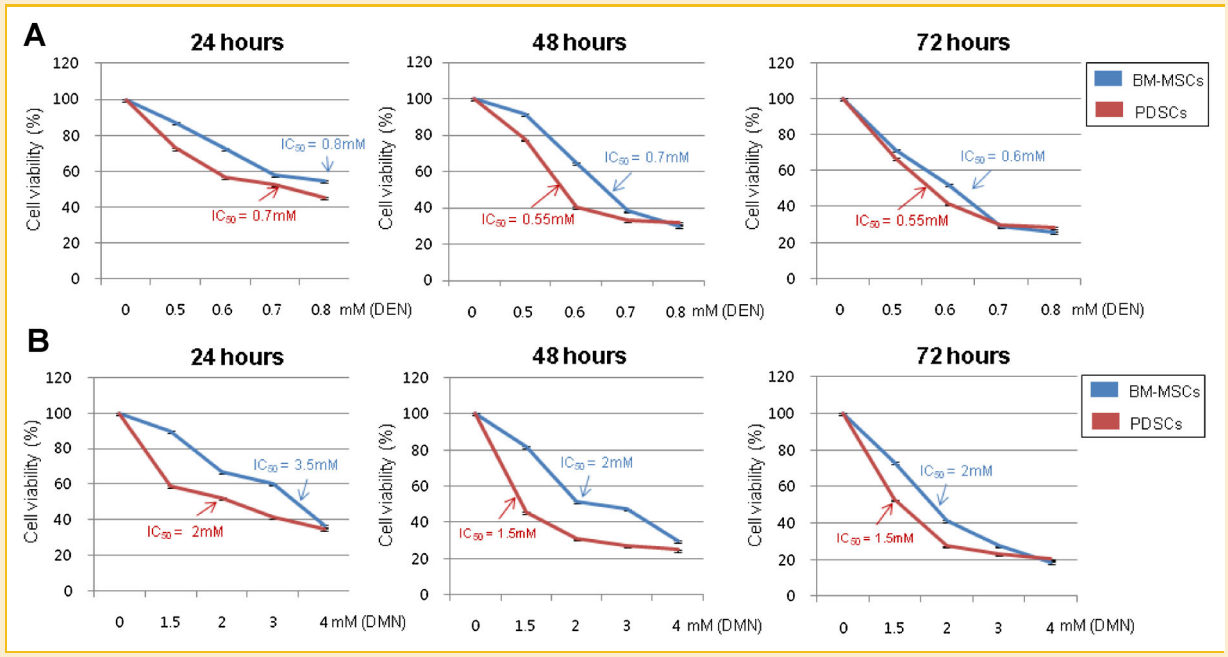

Fig. 3. Comparison of cell viability in BM-MSCs and PDSCs in DEN treatment and DMN treatment for time and dose courses as determined by MTT assays. A: The cell viability of BM-MSCs and PDSCs in DEN treatment after 24, 48, and 72 h. B: The cell viability of BM-MSCs and PDSCs in DMN treatment after 24, 48, and 72 h. The experiments were performed in three independent runs (n = 6). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

(0–4 mM) of DMN for 24, 48, and 72 h (Fig. 3B). As shown in Figure 3B, the values of IC_{50} in BM-MSCs and PDSCs at various time courses gradually decreased. Among these cells, the IC_{50} of DMN was 3.5–2 mM in BM-MSCs, followed by PDSCs at 2–1.5 mM. The tendency for sensitivity to DMN was similar to that of DEN.

Next, we investigated the cytotoxicity of CCl_4 in BM-MSCs and PDSCs at different doses (0–2 mM) for 24, 48, and 72 h (Fig. 4A). As

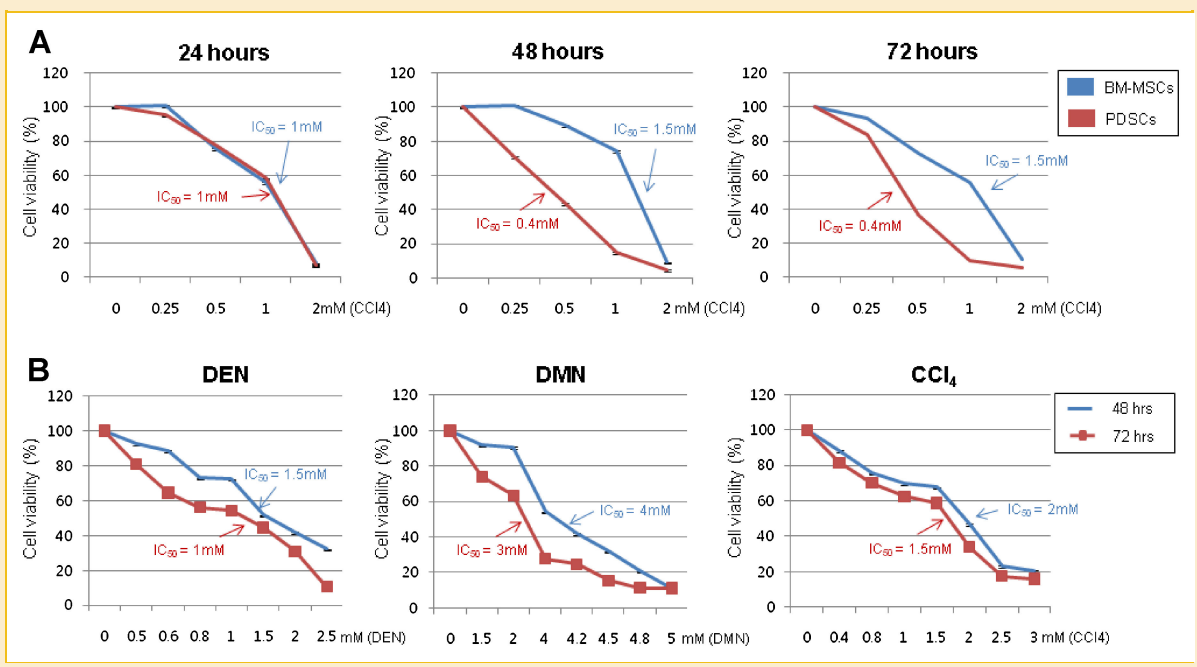

Fig. 4. Comparison of cell viability in BM-MSCs and PDSCs in CCl_4 treatment and the cell viability in PDSCs depends on hepatogenic differentiation to toxicants for time and dose courses as determined by MTT assays. A: The cell viability of BM-MSCs and PDSCs in CCl_4 treatment after 24, 48, and 72 h. B: The cell viability of hepatogenic differentiated PDSCs in DEN, DMN, and CCl_4 treatment after 48 and 72 h. The experiments were performed in three independent runs (n = 6). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

shown in Figure 4A, the cytotoxicity of CCl_4 was similar in each cell type after 24 h. However, the sensitivities changed significantly in PDSCs than BM-MSCs at 48 and 72 h ($P < 0.05$). The IC_{50} of PDSCs was 1–0.4 mM, followed by BM-MSCs which was 1–1.5 mM. In the case of BM-MSCs, the sensitivities in CCl_4 were still lower than PDSCs. These results suggest that PDSCs was more sensitive than BM-MSCs to several toxicants.

We then investigated the cytotoxicity of hepatotoxicants in PDSCs depends on hepatogenic differentiation (Fig. 4B). First, we analyzed whether the cytotoxicity of DEN in PDSCs differed according to hepatogenic differentiation. After hepatogenic differentiation, the value of IC_{50} in PDSCs within 48 and 72 h was 1.5 and 1 mM, respectively. These values of IC_{50} in PDSCs were two to threefold that of naive PDSCs. The cytotoxicity of DEN in hepatogenic differentiation inducing PDSCs was decreased by twofold. These data suggest that the cytotoxicity of DEN differed based on the differences between cells and their hepatogenic differentiation status. Of particular note was that the sensitivity of DEN cytotoxicity in PDSCs was decreased when they were induced to hepatogenic differentiation.

After hepatogenic differentiation, the sensitivity of DMN in PDSCs after 48 and 72 h was decreased to 3 and 4 mM, respectively. The cytotoxicity of DMN in hepatogenic differentiation inducing PDSCs was decreased by twofold. These patterns for sensitivity of DMN in each tested group of cells were similar to the cytotoxicity exhibited by DEN. As in the previous data, the toxicities of CCl_4 were decreased by fourfold in hepatogenic differentiation inducing PDSCs (1.5–2 mM) compared to the undifferentiated cells (0.4 mM; Fig. 4B). These results suggest that PDSCs were more sensitive to the toxicity of CCl_4 than BM-MSCs. In addition, naïve PDSCs were more sensitive than hepatogenic differentiated PDSCs to DEN, DMN, and CCl_4 exposures. Taken together, naïve PDSCs are a useful cell source to assess the toxicity of toxicants such as DEN, DMN, and CCl_4.

We further investigated whether the differences in cytotoxicities of toxicants (DEN, DMM, and CCl_4) in different cell types correlated with expression of the ABC transport system. The ATP transporter system is known to participate in diverse cellular processes, including drug resistance metabolism as well as the transport of various metabolites. It can even manipulate stem cells to enable differentiation into specific lineages [Bunting, 2002; Lin et al., 2006]. Among the ABC transporter systems, the mRNA expression patterns of ABCG1 and ABCG2 differ in BM-MSCs and PDSCs (Fig. 5A). The expression of ABCG1 was only weakly observed in PDSCs and increased in hepatogenic differentiated PDSCs. Although the expression of ABCG2 in PDSCs was lower than that of BM-MSCs, it was increased in hepatogenic differentiated PDSCs. Otherwise, the expression of ABCA2 was different in hepatogenic differentiation inducing BM-MSCs. The expression of ABCA3 was consistent in primitive stem cells and hepatogenic differentiation inducing cells.

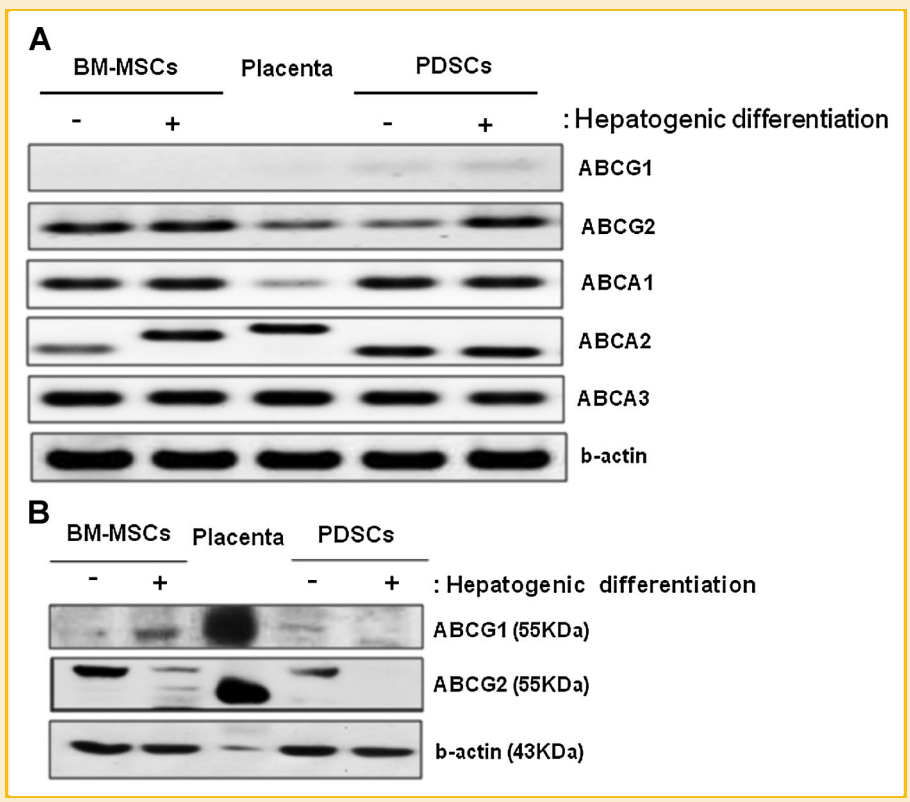

Fig. 5. The expression of the ABC transporter system in BM-MSCs and PDSCs depends on hepatogenic differentiation. A: mRNA levels of ABC transporters in BM-MSCs and PDSCs according to hepatogenic differentiation were evaluated by RT-PCR. B: Protein expression of ABCG1 and ABCG2 in BM-MSCs and PDSCs according to hepatogenic differentiation. Placenta tissues were used as positive controls and β-actin was used as the internal standard.

Otherwise, the expressions of ABCG1 and ABCG2 show the different patterns between BM-MSCs and PDSCs depend on hepatogenic differentiation (Fig. 5B). The ABCG1 expression observed opposite phenotypes depends on hepatogenic differentiation between BM-MSCs and PDSCs, and the expressions of ABCG2 in BM-MSCs and PDSCs were shown a tendency to decrease after hepatogenic differentiation. Decreased ABCG2 expression might be involved in cytotoxicity of PDSCs to toxicants. These results suggest that the expression of ABC transporter systems is involved within cell types and differentiation; therefore, their expression could affect the cytotoxicity of PDSCs.

DISCUSSION

In toxicological research, the development of alternative in vitro toxicity screening systems to replace in vivo screening methods using animal experiments and conventional screening systems is important. Although applications using primary cells derived from rodent embryo or tissue-specific cell lines originating from humans have been tried, the validation of cytotoxicity during cellular biological processing must be improved in order to establish new and alternative in vitro screening methods, and to increase the efficiency of toxicological analysis through these methods [Tiffany-Castiglioni et al., 1999]. Toxicity screening using stem cells is highlighted as an alternative cell source for in vitro toxicity screening because of several limitations of traditional in vitro assays using primary cells or cell lines. For examples, they could not demonstrate the biological process involved in a toxic response to xenobiotics compared to in vivo toxicity testing using animal model. Therefore, these include insufficient cell source and discontinuous characteristics of cellular biological processing which depends on the culture condition.

In a previous report, we demonstrated that endothelial precursor cells derived from mouse embryonic stem cells were more sensitive to 5-FU toxicity than undifferentiated endothelial cells, as well as a mouse endothelial cell line [Kim et al., 2008, 2009]. However, the guideline differentiation technology inducing target tissue-specific cells derived from stem cells had been not established until now. Therefore, optimization and validation for differentiation were required to establish reproducible in vitro toxicity screening methods using stem cells.

For the first time, we have shown that the comparison of BM-MSCs and PDSCs for hepatotoxicity as an in vitro screening system and also PDSCs can be used as a feasible cell source for hepatotoxicity screening regardless of the hepatogenic differentiation steps. Interestingly, PDSCs have the potential for multiple differentiation into several lineages cells including hepatogenic differentiation. In the present study, functions in the hepatogenic-differentiated PDSCs as hepatocytes were also assessed by the ICG uptake assay and urea production. In the cytotoxicity analysis, the sensitivity of the cells toward hepatotoxic chemicals such as DEN, DMN, and CCl_4 showed different patterns. These findings might correlate with the characteristics of chemicals and types of stem cells. Nevertheless, the sensitivity toward hepatotoxic chemicals in naïve PDSCs was sensitive than BM-MSCs. In case of CCl_4 treatment, particularly, naïve PDSCs showed the greatest sensitivity regardless of types of cells (e.g., BM-MSCs) and hepatogenic differentiation.

PDSC is derived from normal term placenta. One of the important functions of the placenta is to transport several factors including nutrients, hormones, O_2, or CO_2 gas and xenobiotics from the mother to the fetus for protection and development [Syme et al., 2004]. In order to maximize the efficacy in transporting system, the ABC transporters play a major role in this process, within the placenta [Aye et al., 2007]. There are seven families of ABC transporters based on structural similarities, named ABC A–G. The ABC transporters, which are expressed in the apical membrane of the placental syncytiotrophoblasts across the placental barrier, use the energy generated by ATP hydrolysis to translocate a broad spectrum of molecules across cell membranes [St-Pierre et al., 2000; Ceckova-Novotna et al., 2006; Serrano et al., 2007]. In addition, ABC transporters are involved in several cellular processes through the strictly regulated transport of various metabolites for cell proliferation, differentiation, cell death, etc. [Lin et al., 2006]. Recently, ABCA3, ABCB1, and ABCG2 were found in primitive stem cells. Of these, ABCG2 was reported as a transporter that operated under all conditions because its function was to recognize small cDNA (homodimer half-transporter) molecules [Sarkadi et al., 2004; Brendel et al., 2007; Mao, 2008]. Also, ABCG2, which is a placenta-specific ABC transporter, was first isolated from the placenta and from multidrug resistant cancer cells and was reported to function as a high capacity drug transporter with wide substrate specificity [Sarkadi et al., 2004; Evseenko et al., 2007]. ABC transporters are known to participate in diverse cellular processes, including drug resistance metabolism as well as the transport of various metabolites. They are also capable of enabling the differentiation of stem cells into specific lineages [Lin et al., 2006]. The expression of ABC transporters depends on the differentiation status of undifferentiated stem cells [Saretzki et al., 2008]. These molecules play a major role in deciding the characteristics of stem cells, as well as the sensitivity of stem cells to toxicants.

In the present report, the expression patterns of ABCG1 in PDSCs with/without differentiation were different than those of BM-MSCs. Although ABCG1 mRNA expression in BM-MSCs was not detected regardless hepatogenic differentiation, protein level of ABCG1 was increased after inducing hepatogenic differentiation. However, the expression of ABCG1 was observed in naive PDSCs and disappeared after hepatogenic differentiation. Otherwise the altered expression of ABCG2 was observed in placental tissues and PDSCs, compared to BM-MSCs in RNA analysis. In spite of that, ABCG2 protein was strongly expressed in BM-MSCs and PDSCs before hepatogenic differentiation. This difference between RNA level and protein expression might be due to post-translational modification during biological processing and the newly function of ABC transporters, which we could not understand until now. These findings suggest that the expression patterns of ABCG1 and ABCG2 might be involved in the sensitivity of PDSCs to several chemicals regardless of hepatogenic differentiation. Therefore, we further study the function of ABC transporters in stem cells research including toxicological field.

In conclusion, naïve PDSCs expressing ABCGs derived by the present method offer possibility for understanding the new

mechanism of ABC transporter in PDSCs for toxicological study and could contribute to the development of a new predictive screening system for the hazard assessment of toxicity using stem cell technology.

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