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ORIGINAL ARTICLE

Simple Maturation of Direct-Converted Hepatocytes Derived from Fibroblasts

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Abstract Target cells differentiation techniques from stem cells are developed rapidly. Recently, direct conversion techniques are introduced in various categories. Unlike pluripotent stem cells, this technique enables direct differentiation into the other cell types such as neurons, cardiomyocytes, insulin-producing cells, and hepatocytes without going through the pluripotent stage. However, the function of these converted cells reserve an immature phenotype. Therefore, we modified the culture conditions of mouse direct converted hepatocytes (miHeps) to mature fetal characteristics, such as higher AFP and lower albumin (ALB) expression than primary hepatocytes. First, we generate miHeps from mouse embryonic fibroblasts (MEFs) with two transcription factors HNF4α and Foxa3. These cells indicate typical epithelial morphology and express hepatic proteins. To mature hepatic function, DMSO is treated during culture time for more than 7 days. After maturation, miHeps showed features of maturation such as exhibiting typical hepatocyte-like morphology, increased up-regulated ALB and CYP enzyme gene expression, down-regulated AFP expressions, and acquired hepatic function over time. Thus, our data provides a simple method to mature direct converted hepatocytes functionally and these cells enable them to move closer to generating functional hepatocytes.

Keywords Mouse induced hepatocytes · Maturation · Dimethyl sulfoxide · Direct conversion

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1 Introduction

For its high prevalence worldwide and poor long-term prognosis, such as premature deaths from liver failure, cirrhosis and hepatocellular carcinoma (HCC), liver disease is considered a serious public health problem [1]. Furthermore, genetic disorders or injuries prevent the liver from performing a broad spectrum of functions such as storage, detoxification, and the production of bile and clotting factors. They also derange metabolic activities, resulting in life-threatening and end-stage liver disease that require liver transplantation eventually. Therefore, the generation of large quantities of hepatocytes raises major concerns for scientists and clinicians. Introduction of

lineage-specific transcription factors (TFs) enabled the direct conversion of somatic cells into adult stem cells, and the direct conversion process is relatively simpler and faster than that of induced pluripotent stem cell (iPSC) generation [2–4]. Recent studies demonstrated the generation of hepatocyte-like cells, induced hepatocytes (iHeps), from mouse and human fibroblasts by forced expression of different TF combinations [5–9]. However, introduction of hepatocyte-specific properties induction in iHep cells lack the specific functions compared to primary hepatocytes. Evidence of expression of markers, ALB (mature hepatocyte) and alpha-fetoprotein (AFP) (immature hepatocytes) was noticed but insufficient to carry out specific roles as hepatocytes. Hence, a number of studies were carried out to ameliorate the function of iHep cells for effectiveness in both the *in vivo* and *in vitro* setting [10, 11].

This is the first study to report that direct converted hepatocytes are matured using not TF forced expression but DMSO treatment. Until now several reports showed the simultaneous maturation on hepatocyte generation step from pluripotent stem cells, but our method is post maturation of direct converted hepatocytes with DMSO [12–14]. Therefore, we sought to determine whether the simple maturation process after direct conversion into hepatocyte-like cells from fibroblasts enhance functionality, and enable them to move closer to the generation of functional hepatocytes.

2 Materials and methods

2.1 Isolation of mouse embryonic fibroblasts

For MEF isolation, uteri of 13.5-day-pregnant mice (Koatech, Pyeongtaek) were isolated, and head and visceral tissues were removed. The remaining bodies were minced, and washed with PBS. Cells were harvested by centrifugation (1200 rpm for 5 min at 4 °C) and resuspended in DMEM containing 10% FBS (Gibco and 1% Penicillin/streptomycin. The cells were cultured on 100 mm dishes until fibroblasts spreading out at 37 °C with 5% CO_2. In this study, we used MEFs within four passages to avoid replicative senescence. Pregnant mice housed under specific pathogen-free conditions in accordance with the Principles of Laboratory Animal Care and the Guide for the Use of Laboratory Animals of Hanyang University (2015-0240A).

2.2 Generation and maturation of mouse direct converted hepatocytes

Generation of miHeps through direct conversion has been shown in previous papers [5–9]. Briefly, 5×10^4 mouse embryonic fibroblasts (MEFs) were transformed by pMX retroviruses containing the hepatic transcription factors Hnf4a and Foxa3. After 48 h, the cells were further cultured in with Dulbecco's modified Eagle's medium F-12 (11965, Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, NY, USA), 10 mM nicotinamide (Sigma-Aldrich, MO, USA), 0.1 M dexamethasone (Sigma-Aldrich), 1% Insulin-Transferrin-Selenium-X Supplement (Gibco), 1% penicillin/streptomycin (Gibco), 20 ng/ml hepatocyte growth factor (Peprotech, NJ, USA) and 20 ng/ml epidermal growth factor (Peprotech) at 37 °C in a CO2 incubator. The medium was changed every day. For maturation of miHeps, cells were cultured in maturation medium contained 2% DMSO in growth medium for more than 7 days. Mature medium was changed every other day.

2.3 Real-time polymerase chain reaction

Total RNA was isolated using Trizol reagent (Gibco). Next, 1 μg RNA samples were reverse transcribed with a Transcriptor First Strand cDNA Synthesis Kit (Roche, IN, USA), and real-time polymerase chain reaction (qPCR) was performed using 10 μL of qPCR PreMix (Dyne Bio), 1 μL of cDNA, and oligonucleotide primers on a CFX Connect Real-Time PCR Detection System (Bio-Rad, CA, USA). Reactions were analyzed in triplicate for each gene; hepatic marker genes primers were as follows; Albumin (forward: 5′-GGCTACAGCGGAGCAACTGA-3′, reverse: 5′-GCCTGAGAAGGTTGTGGTTGTG-3′), AFP (forward: 5′-AGCCTGAACTGACAGAGGAGCA-3′, reverse: 5′-TAAACGCCCAAAGCATCACG-3′), CYP3a13 (forward: 5′-TCCTGCAGAACTTCACTGTCCA-3′, reverse: 5′-TGGTTTCTGGTCCACAGGATACA-3′), CYP2a5 (forward: 5′-GCACTTCCTAGATGACAAGGGACA-3′, reverse: 5′-CAGGCTCAACGGGACAAGAA-3′). The PCR cycles consisted of 40 cycles of 95 °C for 20 s and 60 °C for 40 s. Melting curves and melting peak data were obtained to characterize PCR products.

2.4 Immunostaining

For immunostaining, the cells were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS, pH: 7.4) for 20 min at room temperature. The fixed cells were washed twice with a staining solution of PBS supplemented with 1% fetal bovine serum for 5 min, then permeabilized with 0.25% Triton X-100 for 30 min at room temperature. Cells were then incubated with the following primary antibodies overnight at 4 °C: anti-albumin, hepatocyte Specific Antigen antibody (Hep Par-1), CYP1A2, ASGR1, HNF4a, E-Cadherin. After incubating with the primary antibody, the cells were washed three times with staining solution and incubated with the appropriate fluorescently

labeled Alexa-Fluor secondary antibody for 2 h at room temperature in the dark. Nuclei were counterstained with Hoechst 33342 (Invitrogen).

2.5 Indocyanin green uptake

For the indocyanine green (ICG) uptake assay, the cells were incubated with DID indocyanine green Inj (Dongindang Pharmaceutical) at room temperature for 15 min, and washed three times with PBS. The final concentration of ICG solution was 1 mg/ml. Images were acquired using an Olympus CKX41 microscope with a Canon EOS 600D camera.

2.6 PAS staining

Periodic acid-Schiff (PAS) staining was performed using the Periodic acid-Schiff stain kit (Abcam, Cambridge, UK). Briefly, cells were fixed with 4% paraformaldehyde in PBS, rinsed in slowly running tap water, and then exposed to periodic acid solution for 5 min at room temperature. After rinsing four times with distilled water, the cells were treated with Schiff's reagent for 15 min at room temperature and washed three times with distilled water. Thereafter, the cells were stained with hematoxylin (Modified Mayer's) for 2 min and washed three times with distilled water. The bluing reagent was applied for 30 s to clearly identify the stained cells. Images were acquired using an Olympus CKX41 microscope with a Canon EOS 600D camera.

2.7 Microarray analysis

Total RNA samples were extracted with Trizol Reagent (Gibco), and provided to LAS, Inc (LAS, Korea) for analysis. Briefly, Mouse Ref-8v3 Sentrix bead chips were used (Illumina, San Diego, CA, USA), and normalized by the quantile normalization method across all samples. Arrays were scanned using an Illumina Bead Array Reader Confocal Scanner. Genes were clustered with Cluster 3.0, and heat maps were generated with Tree View 3.0.

2.8 LIVE/DEAD staining

The live/dead viability/cytotoxicity kit for mammalian cells (Invitrogen, USA) was used to measure cell viability. According to the manufacturer's protocol, the cells were double-stained with two probes (calcein AM for live cells, Ethidium Homodier-1 for dead cells) capable of determining live and dead cells. Briefly, 4 uM Calcein AM and 5 uM Ethidium Homodier-1 (EthD-1) was added to the cells. Then, it was cultured at room temperature for 30 min in the dark. Images were acquired using an Olympus CKX41 microscope with a Canon EOS 600D camera.

3 Results

3.1 Generation and characterization of miHeps

To generation of miHeps, mouse embryonic fibroblasts (MEFs) were isolated from E13.5 embryo, and then Foxa3 and HNF4a were virally transduced. After 10-12 day fibroblasts were converted into epithelial-like colonies (Fig. 1A). To elucidate hepatic characteristics of epithelial-like colonies, we conducted immunofluorescence staining with hepatic markers such as ALB, Hep Par-1, CYP1a2, ASGR1, HNF4a and E-Cadherin. As shown in Fig. 1B, each hepatic marker proteins were highly expressed in their epithelial-like colonies, but not in fibroblasts (Fig. S1). Also, according to many reports, pluripotent stem cell-derived hepatocytes expressed increased amount of alpha-fetoprotein (AFP) which is highly expressed in fetal hepatoblasts, but not in adult hepatocytes [14]. This means pluripotent stem cell-derived hepatocytes are immature. To confirm AFP expression in miHeps, ALB and AFP expressions were compared with mouse primary hepatocytes using qRT-PCR measurement. ALB, and AFP expression in miHeps were 100 fold lower and 1000 fold higher than mouse primary hepatocyte (mPH), respectively (Fig. 1C). These results suggest that hepatocyte-like cells can be generated from fibroblasts with two transcription factors, HNF4alpha and Foxa3, and these cells represent immature characteristics.

3.2 Maturation of miHeps

Generally, Dimethyl sulfoxide (DMSO) was used to differentiate and enrich hepatocyte-like cells from pluripotent stem cells [20], and mature of HpaRG cells, a human hepatocellular carcinoma cell line [21]. Therefore, for maturation of miHeps, we added 2% DMSO in the culture media. With addition of DMSO, sequential morphologic change of miHeps were induced from day 5 to day 20 compare to untreated miHeps (Fig. 2A). After day 20, DMSO-treated miHeps morphology was showing matured hepatocytes shape, which is abundant cytosol, small nuclei and clear cell to cell junction. Also, hepatic marker gene expressions were evaluated by qRT-PCR. ALB, Cyp3a13 and Cyp2a5 expressions were elevated more than two folds by DMSO treatment (Fig. 2B). Additionally AFP and fetal hepatoblasts marker expressions were down-regulated. To confirm correlation between gene expression and hepatic function, indocyanine green (ICG) uptake and Periodic

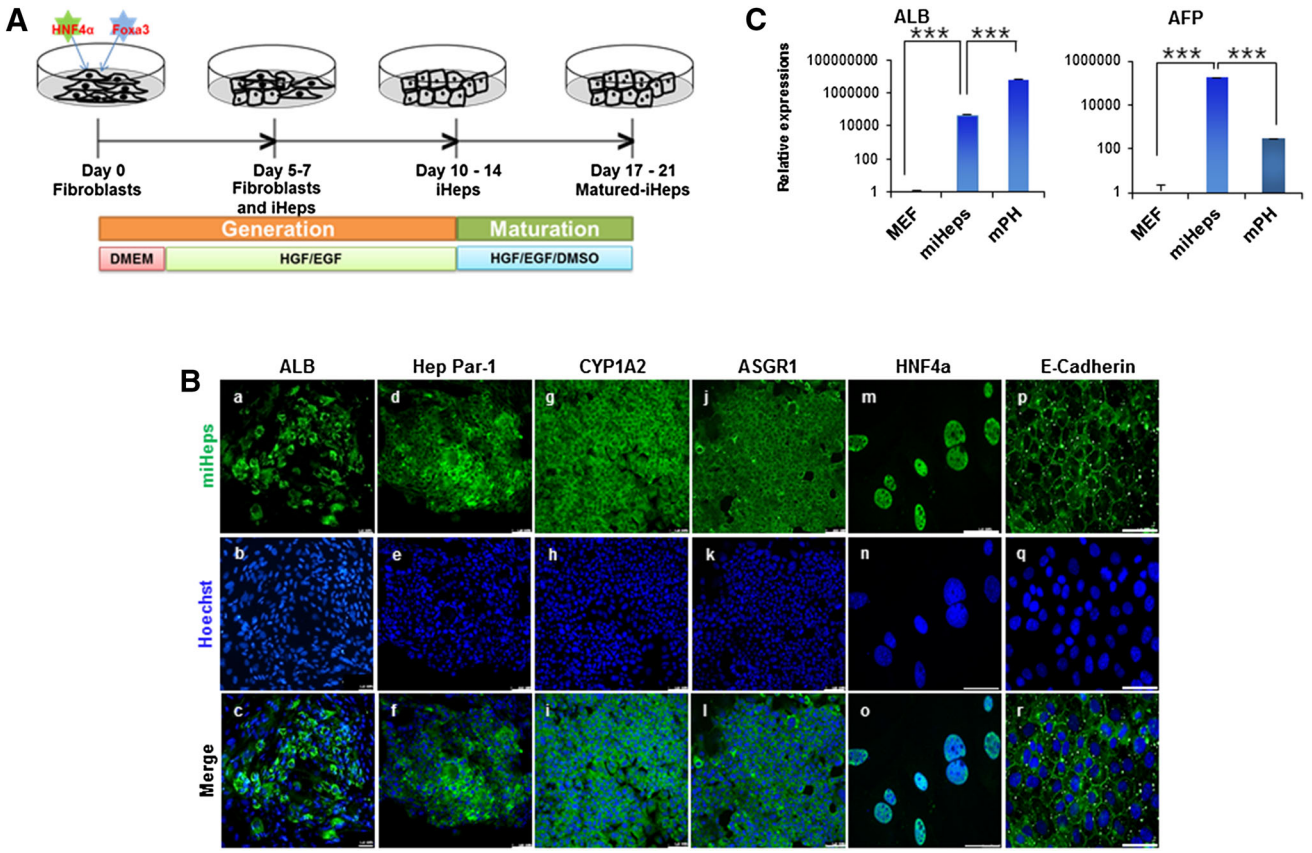

Fig. 1 Generation and characterization of mouse iHeps. **A**. A schematic overview of generation and maturation for 21 days. After generation of miHeps for 14 days, 2% DMSO is treated to mature hepatic function. **B**. Immunocytochemistry of hepatic marker proteins. Each hepatic marker proteins are expressed in miHeps before maturation (*a*, *d*, *g*, *j*, *m*, *p*). Hoechst (*Blue*) labels all nuclei (*b*, *e*, *h*, *k*, *n*, *q*). *Scale bar* 100 um. **C**. Quantitative real-time PCR of albumin (ALB) and alpha-feto protein (AFP). Data are mean ± SD. $*p < 0.05$; $**p < 0.01$; $***p < 0.005$

acid-Schiff (PAS) staining were conducted. As expected, increased ICG uptake and glycogen storage were detected in matured miHeps (Fig. 2C). It was shown that the hepatic gene expressions and function were increased by continuous DMSO treatment.

3.3 Cell death and methylation status of miHeps

Hepatoblasts are continuously growing with cell division in fetal period, but adult hepatocytes are stopped their cell division. To confirm cells proliferation, we calculated cell number every other day. The miHeps without DMSO drastically proliferated until day 8, but after that point cell numbers were gradually decreased. While DMSO-treated miHeps did not proliferated, and induced mature morphology (Fig. 3A). To investigate the cause of decline of cell number, each miHeps were stained with Calcein AM (green) and Ethidium Homodimer-1 (EthD-1, red) for confirmation of cell death on day 14. DMSO-untreated miHeps were stained with EthD-1, which means cell death, but matured miHeps were barely stained with EthD-1 (Fig. 3B). On the contrary to this, Calcein AM was stained more in matured miHeps. It is suggests that DMSO treatment matured miHeps without cell death.

DNA methylation is very important mechanism that controls of development, maturation and aging. In the liver, drug metabolism genes, cytochrome P450, is activated through decrease of histone-3-lysine-4 trimethylation (H3K4me3) and histone-3-lysine-27 trimethylation (H3K27me3) in promoter region [25]. To assess the relationship between hepatic maturation and epigenetic regulation, we detected several histone-3-lysine methylation status with or without DMSO by Western-blot. Surprisingly, histone-3-lysine-4, 9, 36, 79 trimethylation status was down-regulated in matured miHeps (Fig. 3C). While, H3K27me3 was not detected (Data not shown). And we found increased lysine-specific demethylase (Lsd1) protein, one of histone demethylase (Fig. 3C). These results suggest that Lsd1 is up-regulated, and demethylate histone-3-lysine directly or indirectly in matured miHeps. Therefore, as DMSO adding in culture media, we can easily obtained matured miHeps.

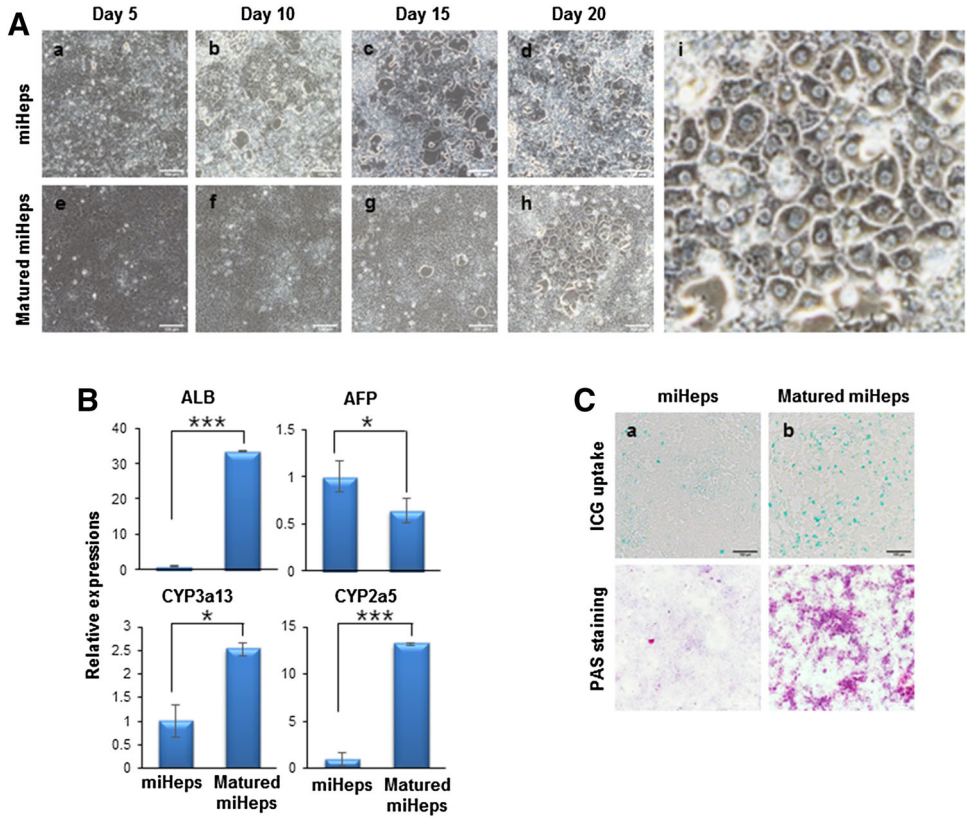

Fig. 2 Analysis of matured hepatic characteristics of miHeps with or without DMSO. **A**. Representative images at different time point between miHeps (*a*, *b*, *c*, *d*) and matured miHeps (*e*, *f*, *g*, *h*). Ai shows a magnified image of Ah. *Scale bar* 100 um. **B**. Relative gene expressions for hepatic markers. Data are mean ± SD. $*p < 0.05$; $**p < 0.01$; $***p < 0.005$. **C**. Representative pictures showing ICG uptake (*a*, *b*) and PAS staining (*c*, *d*) in cells. *Scale bar* 100 um

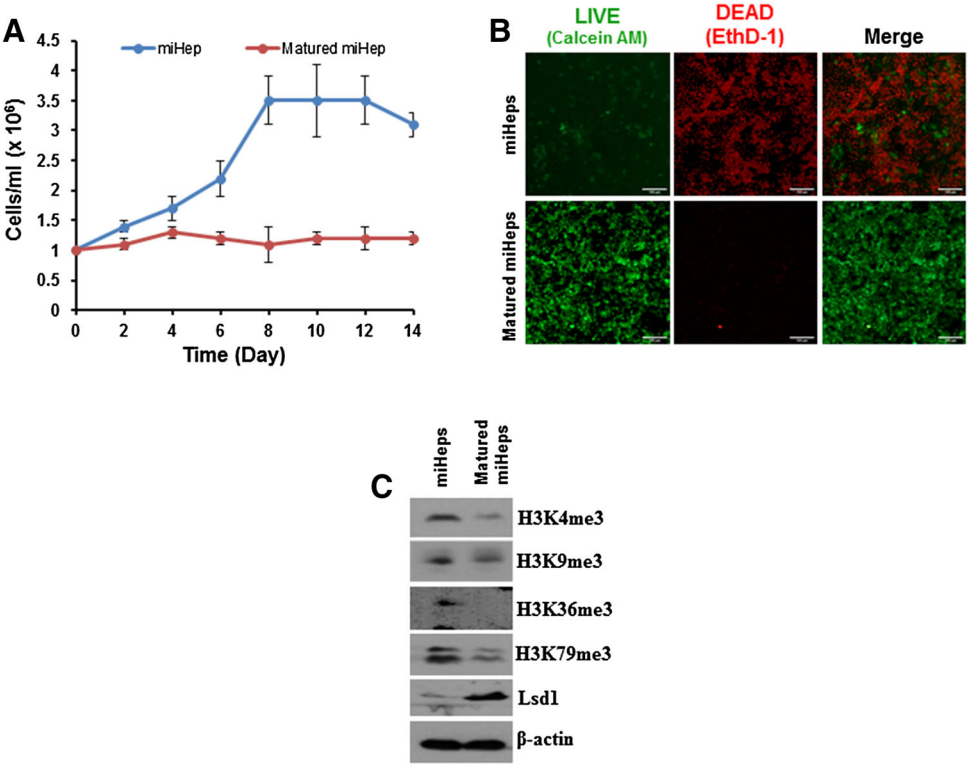

Fig. 3 Analysis of cell death and methylation status of histone 3 lysines in matured miHeps. **A**. Growth curve of both miHeps and matured miHeps. **B**. LIVE/DEAD staining for cell death measurement. Calcein AM, Calcein Acetoxymethyl; Etdh-1, Ethidium Homodimer-1. *Scale bar* 100 um. **C**. Western blot for methylation status of histone 3 lysines. H3K4me3, Tri-methylation of lysine 4 on histone H3; H3K9me3, Tri-methylation of lysine 9 on histone H3; H3K36me3, Tri-methylation of lysine 36 on histone H3; H3K79me3, Tri-methylation of lysine 79 on histone H3; LSD1, lysine (K)-specific demethylase 1A

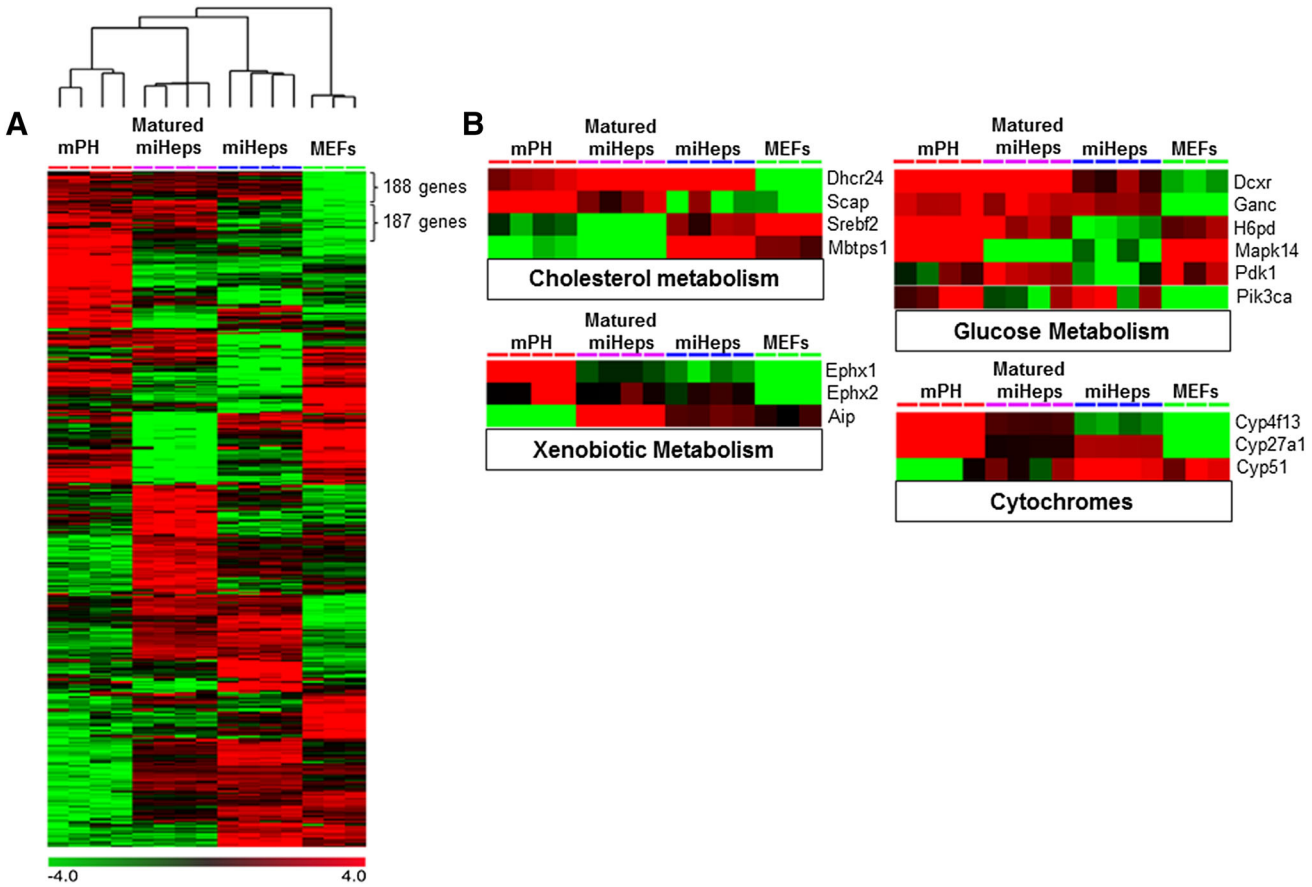

Fig. 4 Gene expression profiles of miHeps, matured miHeps and hepatocytes. **A**. Heat map of the genes up-or down-regulated in miHeps and hepatocytes. The colors represent up-regulation (*Red*) and down-regulation (*Green*). **B**. Heat map analysis of different metabolic pathways

3.4 Global gene expression of miHeps

We analyzed global gene expression in the miHeps and matured miHeps by microarray analysis. The transcriptional profiles of the miHeps and matured miHeps clustered with that of adult hepatocyte, but was separated from that of MEFs. It indicates that the matured miHeps were closer to hepatocytes than miHeps and MEFs (Fig. 4A). These miHeps and matured miHeps expressed genes were related to several metabolic pathway which represent hepatic functions, metabolism of cholesterol, glucose and xenobiotic and cytochrome expression (Fig. 4B). Especially, we found 188 genes or 187 genes, which is commonly expressed between all three samples and matured matured miHep and hepatocytes, respectively, respectively (Fig. 4A, Table. S2). To confirm the functional features of these genes, gene ontology (GO) analysis was conducted using ClueGo, a Cytoscape plug-in software, and a list of categorized genes (Fig. S2). The terms of 188 genes and 187 genes obtained represented several types of disease and metabolism. In addition, several metabolism genes, such as Cyp27a1, Dhcr24, EphX1, Ganc and Pdk1, were included in 187 genes (Fig. 4B, Table S2 with bold). The results indicate that the matured miHeps expressed more hepatic functional genes, and related more closely to hepatocytes. This study is believed to be the first study to use DMSO treatment for maturation of directed converted hepatocyte.

4 Discussion

Patients with end-stage liver disease suffer significantly and need definite treatment such as liver transplantation for improved prognosis [15, 16]. Unfortunately, the demand for liver transplantation is outstripping potential donors [17]. Therefore, vast interest is focused on the generation of functional hepatocytes independent of donor liver organs [18, 19]. In this study, to solve these problems, we suggested direct converted hepatocyte from fibroblasts, and simple maturation methods with DMSO treatment. In our results, functional hepatic marker genes, ALB, Cyp3a13 and Cyp2a5, were elevated, while AFP expressions were down-regulated with DMSO treatment (Fig. 2B). According to previous reports, DMSO was used to culture hepatocytes in

the long term [20], mature of HepaRG cells [21], and enrich hepatic cells from pluripotent stem cells [22]. Also, DMSO induces reversible G1 arrest by retinoblastoma activation [23]. Therefore, matured miHeps were not proliferated by DMSO treatment without cell death (Fig. 3A, B).

In the developmental stage of the liver, CYP genes are regulated by epigenetic changes [24]. Recently, it has been reported that DNA methylation is related to hepatic gene expression in fetal and adult hepatocyte [25]. Furthermore, some CYP genes are regulated by DNA methyltransferases (DNMTs) and histone deacetylase (HDACs) during hESCs differentiation into hepatocytes [26]. Inhibition of DNMTs and HDACs increase several CYP genes expression. Also, 5-azacytidine, which is a known DNA methylase inhibitor, enhances the hepatic function of HP14.5, hepatic progenitor cells [27]. In our results, the trimethylation status of several histone H3 lysines was decreased by the maturation of miHeps. H3K4me3, H3K9me3, H3K36me3 and H3K79me3 were decreased (Fig. 3C), but H3K27me3 was not detected (data not shown). Therefore, it can be inferred that the maturation of miHeps by DMSO treatment induces epigenetic regulation.

Interestingly, Lsd1, a histone demethylase, was increased in matured miHeps (Fig. 3C). LSD1 can demethylate me1 and me2 of lysine 4 and 9 (H3K4 and H3K9) of histone 3 [28]. LSD1-p53 interaction represses alpha-fetoprotein (AFP) transcription during liver regeneration after birth [29]. It is a reversible reaction whereby AFP is reactivated by the elimination of p53. Taken together, in this report, we have shown the demethylation of several histone 3 lysines and LSD1 expression as mechanisms of hepatic maturation of miHeps. Although these mechanisms require more investigation, we strongly suggest that this is the first study to successfully demonstrate the beneficial effect of maturation by DMSO treatment.

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Compliance with ethical standards

Conflict of interest The authors that they have no conflict of interest.

Ethical standards Pregnant mice housed under specific pathogen-free conditions in accordance with the Principles of Laboratory Animal Care and the Guide for the Use of Laboratory Animals of Hanyang University (2015-0240A).

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