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Original Article

Generation of high quality of hepatocyte-like cells from induced pluripotent stem cells with Parp1 but lacking c-Myc

Chi-Shuan Huang [a], Hsin-Chi Lin [b,c], Kai-Hsi Lu [c], Wai-Wah Wu [b], Ya-Chi Yang [d], Yi-Ping Yang [d], Chih-Hung Chiang [e,f], Jung-Hung Hsieh [e,f], Yuh-Lih Chang [d,e,g,**], Shou-Dong Lee [b,e,*]

[a] *Division of Colorectal Surgery, Department of Surgery, Cheng-Hsin General Hospital, Taipei, Taiwan, ROC*
[b] *Division of Gastroenterology, Department of Internal Medicine, Cheng-Hsin General Hospital, Taipei, Taiwan, ROC*
[c] *Department of Medical Research and Education, Cheng-Hsin General Hospital, Taipei, Taiwan, ROC*
[d] *Institute of Pharmacology, National Yang-Ming University, Taipei, Taiwan, ROC*
[e] *School of Medicine, National Yang-Ming University, Taipei, Taiwan, ROC*
[f] *Division of Urology, Department of Surgery, Taipei Veterans General Hospital and Su-Ao & Yuan-Shan Branch, Taipei, Taiwan, ROC*
[g] *Department of Pharmacy, Taipei Veterans General Hospital, Taipei, Taiwan, ROC*

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Abstract

Background: Induced pluripotent stem cells (iPSCs) have a great potential for application in patient-specific therapy. The reprogramming method that does not involve c-Myc reduces tumorigenic risk, but also largely reduces the efficiency of generation of iPSCs, especially for those reprogrammed from damaged cells. Poly(ADP-ribose) polymerase 1 (Parp1) catalyzes a reaction of poly(ADP-ribosylation) and has been reported to enhance cell reprogramming.
Methods: Using Oct-4/Sox2/Klf4/Parp1 (OSKP) reprogramming method, reprogramming factors plus Parp1 were capable of generation of iPSCs from adult fibroblasts and further toward to differentiate from iPSCs status into hepatocyte-like cells.
Results: Our results showed that Oct-4/Sox2/Klf4/Parp1 (OSKP)-derived iPSC exhibited regular pluripotent properties, long-term passages and more stable cellular-divided period. These OSKP-derived iPSCs can effectively differentiate into hepatocyte-like cells (OSKP-iPSC-Heps), and present high mRNA levels of Sox17, HNF3b, and HNF4a in OSKP-iPSC-Heps. The mature hepatic functions, including CYP3A4, LDL uptake, glycogen synthesis and urea secretion were analyzed and well detected in OSKP-iPSC-Heps on day 14 post-differentiation.
Conclusion: In conclusion, we demonstrated that Parp1 promoted reprogramming process to generate the high quality of iPSCs, which could be used as a high quality source of hepatocytes.

Keywords: Hepatocyte; Induced pluripotent stem cells; Poly(ADP-ribose) polymerase 1

1. Introduction

Orthotopic liver transplantation has shown efficacy in the treatment of end-stage liver failure, liver cirrohsis[1−3] and Non-alcoholic fatty liver disease (NAFLD).[4] However, high cost, donor organ shortage and life-long immunosuppressive medications limit the availability of such treatment.[5] Recently, transplantation of mesenchymal stem cells in mice resulted in functional engraftment[6] and remarkable therapeutic efficacy[7] in recipients affected by NASH, suggesting that cell-based therapy

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

* Corresponding author. Dr. Shou-Dong Lee, Division of Gastroenterology, Department of Internal Medicine, Cheng-Hsin General Hospital, 45, Cheng-Hsin Street, Taipei 112, Taiwan, ROC.
** Corresponding author. Dr. Yuh-Lih Chang, Department of Pharmacy, Taipei Veterans General Hospital, 201, Section 2, Shi-Pai Road, Taipei 112, Taiwan, ROC.
E-mail addresses: ylchang@vghtpe.gov.tw (Y.-L. Chang), ch9318@chgh.org.tw (S.-D. Lee).

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can be applied as a feasible alternative to treatment of this disease. However, the molecular and cellular mechanisms of NASH are still not fully understood. Therapeutic strategies that target specific pathways in the pathogenesis are urgently needed.

Recent progress in induced pluripotent stem cell (iPSC) study has demonstrated that somatic cells can be reprogrammed into a pluripotent state by forced expression of Yamanaka's four factors.[8−11] This novel technology has raised the possibility of personalized therapy, using patient-specific iPSCs. However, senescence is a critical barrier that may limit reprogramming. To overcome this obstacle, Lapasset et al. have reported a protocol using a combination of six factors, Oct4/Sox2/Klf4/c-Myc/Lin28/Nanog, to improve iPSC reprogramming efficiency of senescent fibroblasts derived from aged donors.[12] However, this protocol uses c-Myc, which is an proto-oncogene that has been linked to the risk of tumorigenesis, and therefore it may hinder its clinical application.[13] Although avoiding the use of c-Myc gene reduced the tumorigenic incidence, the reprogramming efficiency was concomitantly suppressed.[14,15] As can be expected, the use of such c-Myc-free protocol may have particularly low efficiency in reprogramming of senescent cells. Therefore, it remains an open question how to efficiently increase reprogramming rate and pluripotency of senile tissues/fibroblasts and simultaneously avoid the undesired effects induced by exogenous c-Myc transduction.

Poly(ADP-ribose) polymerase 1 (Parp1), enzyme that catalyzes PARylation, is a key effector involved in DNA repair, replication, transcription and genomic methylation.[16,17] It has been observed that hepatocytes from Parp1-deficient mice have DNA damage and decreased proliferative responses to mitogens.[18] Malfunction of Parp1 signaling may exacerbate diet-induced obesity and insulin insensitivity.[19] These findings suggested that Parp1 is a crucial factor in hepatic protection. Recently, in the work of Doege et al. and in our previous work, it was demonstrated that Parp1 can promote reprogramming,[20,21] particularly in the absence of c-Myc gene.[21] Hence, it will be important to investigate whether Parp1 could also enhance iPSC generation from senescent somatic cells. Whether such resultant iPSCs, generated by Parp1-mediated reprogramming, can be employed as a cell source of high qualities of hepatocyte generation, remains an open question.

In the present study, we demonstrated that Parp1 can enhance iPSC generation from somatic cells in the presence of three other reprogramming factors Oct4/Sox2/Klf4 (OSKP). After induction of hepatic differentiation into hepatocyte-like cells (iPSC-Heps), these OSKP-iPSC-Heps expressed liver-specific markers and characteristics, exhibited mature hepatocyte functions. Our data may facilitate development of safe protocol for the patient-specific cell therapy of elderly patients with liver failures.

2. Methods

2.1. Generation of iPSC cell lines and differentiation protocols

iPSCs were generated from skin fibroblasts derived from the normal donors by the transduction of retroviral vectors encoding four transcription factors (Oct-4/Sox2/Klf4/c-Myc; OSKM), three transcription factors (Oct-4/Sox2/Klf4; OSK), or three factors plus Parp1 (Oct-4/Sox2/Klf4/Parp1; OSKP), as described previously.[15] Total of 12 clones (Re-1 to Re-12; OSKM) were selected and established. Undifferentiated iPSCs were routinely cultured and expanded on mitotically-inactivated MEFs (50,000 cells/cm2) in six-well culture plates (BD Technology) in the presence of 0.3% leukemia inhibitory factor in an iPSC medium consisting of Dulbecco's Modified Eagle's Medium (DMEM; Sigma), supplemented with 15% fetal bovine serum (FBS; Invitrogen), 100 mM minimal essential medium (MEM) nonessential amino acids (Sigma), 0.55 mM 2-mercaptoethanol (Gibco), and antibiotics (Invitrogen), and 0.3% leukemia inhibitory factor. Every three to four days, colonies were detached with 0.2% collagenase IV (Invitrogen), dissociated into single cells with 0.025% trypsin (Sigma−Aldrich) and 0.1% chicken serum (Invitrogen) in PBS, and replated onto MEFs. For embryoid body (EB) formation, iPSCs were dissociated into a single cell suspension by 0.25% trypsin−EDTA and plated onto non-adherent culture dishes in DMEM with 15% FBS, 100 mM MEM nonessential amino acids, 0.55 mM 2-mercaptoethanol and antibiotics at a density of 2×10^6 cells/100 mm plate. After 4 days in floating culture, EBs were transferred onto gelatin-coated plates and maintained in the same medium for 24 h. EBs were then induced to differentiate into hepatocyte lineage by using a two-step procedure as previously described.[15] For endoderm induction, iPSCs were incubated for 24 h in RPMI 1640 medium (Invitrogen/Gibco, Rockville, MD, USA), supplemented with 100 ng/ml Activin A (Peprotech). D the following 2 days, 0.1 and then 1% insulin-transferrin-selenium (Invitrogen/Gibco) was added to this medium. Following Activin A treatment, the differentiated iPSCs were cultured in Hepatocyte Culture Medium (HCM) (Cambrex, Baltimore, MD, USA) containing 30 ng/ml FGF4 for 4 days. Then, the differentiated cells were incubated in HCM containing 20 ng/ml HGF for 6 days, in HCM containing 10 ng/ml oncostatin-M (R&D, Minneapolis, MN, USA) plus 0.1 mM dexamethasone (Sigma Aldrich) for 5 days.

2.2. Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Real-time RT-PCR was performed as previously described.[22] For real-time RT-PCR analysis, the total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA). The total RNA (1 μg) from each sample was reverse transcribed, using 0.5 μg of oligo dT and 200 U Superscript II RT (Invitrogen, Carlsbad, CA). The amplification was carried out in a total volume of 20 μl, containing 0.5 μM of each primer, 4 mM $MgCl_2$, 2 ml LightCycler FastStart DNA Master SYBR green I (Roche Diagnostics, Pleasanton, CA) and 2 ml of 1:10 diluted cDNA. The quantification of the unknown samples was performed by LightCycler Relative Quantification Software, version 3.3 (Roche Diagnostics). In each experiment, the GAPDH housekeeping gene was amplified as a reference standard. PCR reactions were prepared and performed in

duplicate and heated to 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 55 °C for 5 s, and 40 cycles of extension at 72 °C for 20 s. Standard curves (cycle threshold values versus template concentration) were prepared for each target gene and for the endogenous reference (GAPDH) in each sample.

2.3. Alkaline phosphatase staining

For detecting the alkaline phosphatase (AP) activity of iPSCs, cells were fixed with 80% ethanol and then stained using the Blue Alkaline Phosphatase Substrate kit III (Vector Laboratories) according to the manufacturer's instructions.

2.4. Cellular uptake assay of low-density lipoprotein (LDL)

The cellular uptake capability of 1,1′-dioctadecyl-1-,3,3,3′,3′,-tetramethyl-indo-carbocyanine perchlorate conjugated to acetylated-LDL (DiI-Ac-LDL; AbD Serotec) was determined by fluorescent microscopy. Cells were incubated with 20 μg/ml DiIAC-LDL at 37 °C for 24 h. Incorporation of DiI-Ac-LDL into cells was visualized by fluorescence microscopy.

2.5. Periodic acid-schiff (PAS) staining for glycogen

Cells in culture dishes were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 for 10 min, followed by oxidation in periodic acid (Sigma–Aldrich) for 5 min at room temperature. Cells were rinsed three times in distilled water and treated with Schiff's reagent (Sigma–Aldrich) for 15 min at room temperature, then washed in running tap water for 5 min. Samples were counterstained with Hematoxylin Solution for 90 s.

2.6. MTT assay

For evaluation of cell survival, cells were seeded on 24-well plates at a density of 2×10^4 cells/well, followed by the addition of methyl thiazol tetrazolium (MTT; Sigma). The amount of MTT formazan product was determined by measuring absorbance at 560 nm, using a microplate reader (SpectraMax 250, Molecular Devices, Sunnyvale, CA, USA).

2.7. Immunofluorescence staining

The avidin-biotin complex-based method was used for immunohistochemical staining of differentiated iPSCs. And then following washes with 3% hydrogen peroxide, sodium azide and antigenicities were retrieved using a microwave. Each slide was then treated with antibodies for HNF-3β (Chemicon International, Temecula, CA), Albumin (Chemicon International, Temecula, CA), AFP (Upstate Biotechnology, Waltham, MA, USA). Immunoreactive signals were detected with a mixture of biotinylated rabbit anti-mouse IgG and Fluoresave (Calbiochem, La Jolla, CA, USA) and a confocal microscope (Olympus, FV300).

2.8. Liver functional tests

Biochemical parameters were measured using standard clinical methods. After anesthesia by ketamine (10 mg/100 g body wt), intracardiac aspiration of blood was performed. 0.8–0.9 ml of blood sample was collected from the heart into a pyrogen-free syringe containing ~75 units of heparin sodium. Serum ammonia and biochemical parameters, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin (TBIL) were analyzed using a Vitro DT chemistry system (Johnson & Johnson).

2.9. Statistical analysis

The results are expressed as mean ± SD. Statistical analyses were performed using the *t*-test for comparing two groups, and one-way or two-way ANOVA, followed by Bonferroni's correction, to detect differences among three or more groups. The results were considered statistically significant at $p < 0.05$.

3. Results

3.1. Reprogramming SnFBs into OSKP-iPSCs using OSK and Parp1

It remains an open question whether senescent somatic cells or senescent cell-reprogrammed iPSCs from aged patients could serve as feasible cell source for cell-based therapy in this disease. We have previously demonstrated that Parp1 can promote the reprogramming of somatic cells into iPSCs, particularly in the absence of c-Myc gene.[21] In the present study, isolated skin fibroblasts and reprogrammed them into iPSCs, followed by hepatic differentiation into iPSC-derived hepatocytes (iPSC-Heps). Subsequently, we compared several hepatic-specific characteristics and therapeutic potential of these iPSC-Heps with senescent hepatocytes isolated from the same cell donor. To investigate whether Oct4/Sox2/Klf4 plus Parp1, but not c-Myc, were able to promote reprogramming of senescent somatic cells, we isolated senescent fibroblasts (SnFBs), and examined the ability of different combinations of reprogramming factors to drive their reprogramming into iPSCs. Transfection of Oct4/Sox2/Klf4 (OSK) or Oct4/Sox2/c-Myc (OSM) into SnFBs caused very weak reprogramming efficiency, as compared to the conventional cocktail Oct4/Sox2/Klf4/c-Myc (OSKM). However, addition of Parp1 to OSKM improved efficiency of iPSC generation. Notably, overexpression of Parp1 in the presence of three other factors, but lacking c-Myc (OSKP), promoted iPSC generation at a moderate magnitude (Fig. 1A). These OSKP-reprogrammed iPSCs formed colonies very similar to embryonic stem cells (ESCs) and were positive for alkaline phosphatase (ALP) (Fig. 1B). Quantitative RT-PCR showed that, similarly to mouse ESCs, OSKP-mediated reprogramming substantially increased the gene expression of stemness factors, including Oct4, Sox2, Nanog, and Rex1 (Fig. 1C–F). Furthermore, OSKP-reprogrammed

iPSCs were capable of differentiation into tridermal lineages (mesodermal-lineage, positive for α-smooth muscle actin; neuroectodermal-lineage, positive for nestin; data not shown). These data indicated that Parp1 plus the conventional factors Oct4/Sox2/Klf4 could reprogram senescent somatic cells into pluripotent stem cells.

3.2. Differentiation of OSKP-iPSC into functional OSKP-Heps

The differentiation of OSKP-iPSC towards the hepatocyte (Hep) lineage progresses through two stages, as shown in Fig. 2A, while bFGF, activin and HGF are known to be key regulators in the generation of hepatocytes during differentiation. After shifting of OSKP-iPSC-derived embryoid bodies (EBs) to hepatic differentiation media, they gradually exhibited more spread and cuboidal morphology along the differentiation time course and eventually differentiated into iPSC-Heps (OSKP-iPSC-Heps; Fig. 2B). The human ESC H9-derived EBs also differentiated into hepatocytes through the same stages. The differentiated hepatocyte-like cells of ESC H9 and OSKP-iPSC origin at day 21 exhibited cuboidal epithelial morphology. These observations indicated that Parp1 had a complementary role in the establishment of early reprogramming marks and OSKP-iPSCs possessed hepatic differentiation ability.

3.3. Characterization hepatocytes differentiated from OSKP-iPSCs

In order to characterize the functionality of hepatocyte-like cells derived from OSKP-iPSC, mRNA expression of endodermal marker Sox17, liver-specific markers hepatocyte nuclear factor 3 beta (HNF-3β) and hepatocyte nuclear factor 4 alpha (HNF-4α) in OSKP-iPSC-Hep was compared with that in OSKM-iPSC-Hep and H9-Hep at day 21. Sox17, HGF-3β and HNF-4a were considerably upregulated (Fig. 3). Next, we compared the protein expression of liver enzyme cytochrome P450 3A4 (cyp3A4) at different time points after post-differentiation in OSKP-iPSC-Heps, OSKM-iPSC-Heps and H9-iPSC-Heps (Fig. 4A). The amount of protein expression of Cyp3A4 was gradually increased at both 7 days and 14 days post-differentiation (Fig. 4A). Using a cellular uptake assay for DiI-Acetylated-low density lipoprotein and Periodic acid-Schiff staining, we found that the LDL uptake ability and glycogen synthesis were significantly risen in OSKP-iPSC, OSKM-iPSC and H9 at days 7 and 14 post-differentiation(Fig. 4B and C). Furthermore, using ELISA measuring urea secretion, we found that OSKP-iPSC, OSKM-iPSC and H9 secreted a higher level of urea at day 14 than at previous differentiation day (Fig. 4D). The flow cytometry results of albumin expression level confirmed prominent differentiation of OSKP-iPSC and H9 into hepatocyte-like cell

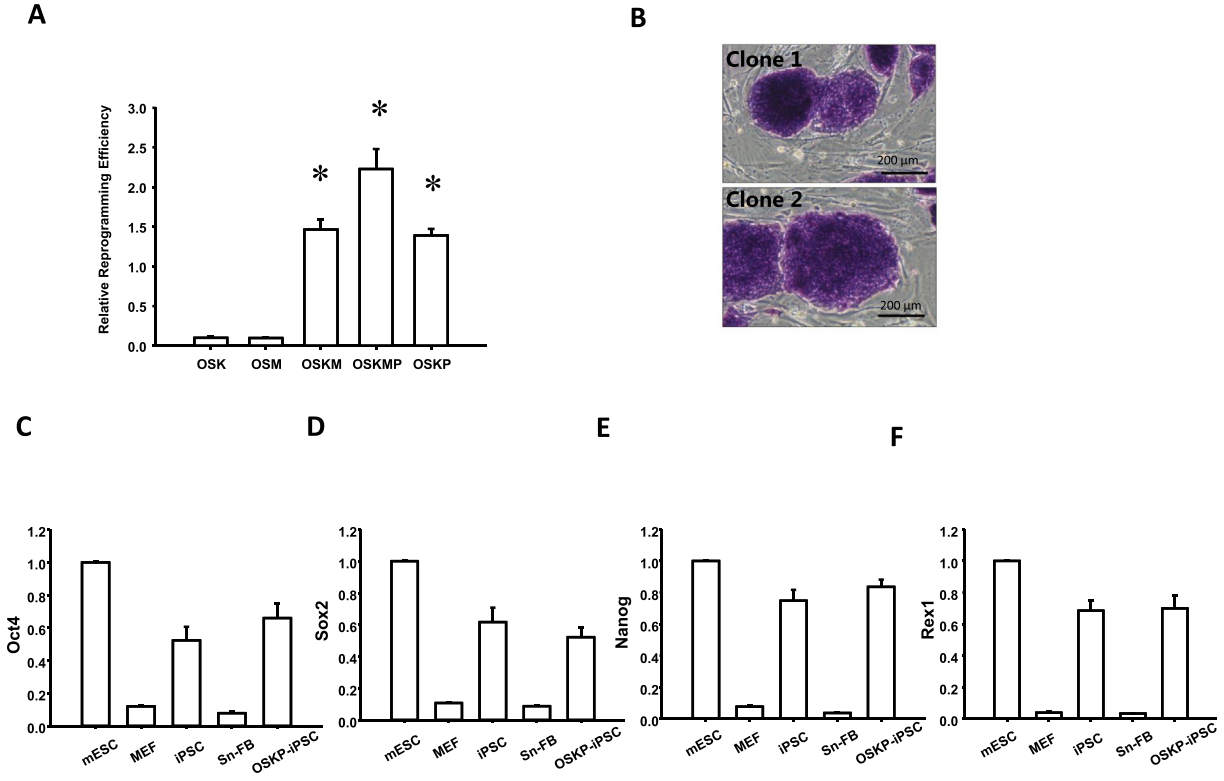

Fig. 1. **Reprogramming senescent somatic cells into iPSCs using Oct4/Sox2/Klf4 and Parp1.** (A) Relative reprogramming efficiency among iPSCs generated by different reprogramming factors, including Oct4/Sox2/Klf4/Parp1, Oct4/Sox2/Klf4/c-Myc, Oct4/Sox2/Klf4, Oct4/Sox2/c-Myc, and Oct4/Sox2/Klf4/c-Myc/Parp1. (B) Morphology of Oct4/Sox2/Klf4/Parp1 iPSCs with AP staining on two iPS cell. (C–F) Relative mRNA expression levels of Oct4, Sox2, Nanog and Rex2 among mouse embryonic stem cells (mESCs), MEF, Oct4/Sox2/Klf4/c-Myc-reprogrammed iPSCs, senescent fibroblasts, and Oct4/Sox2/Klf4/Parp1-reprogrammed iPSCs. *$p < 0.05$.

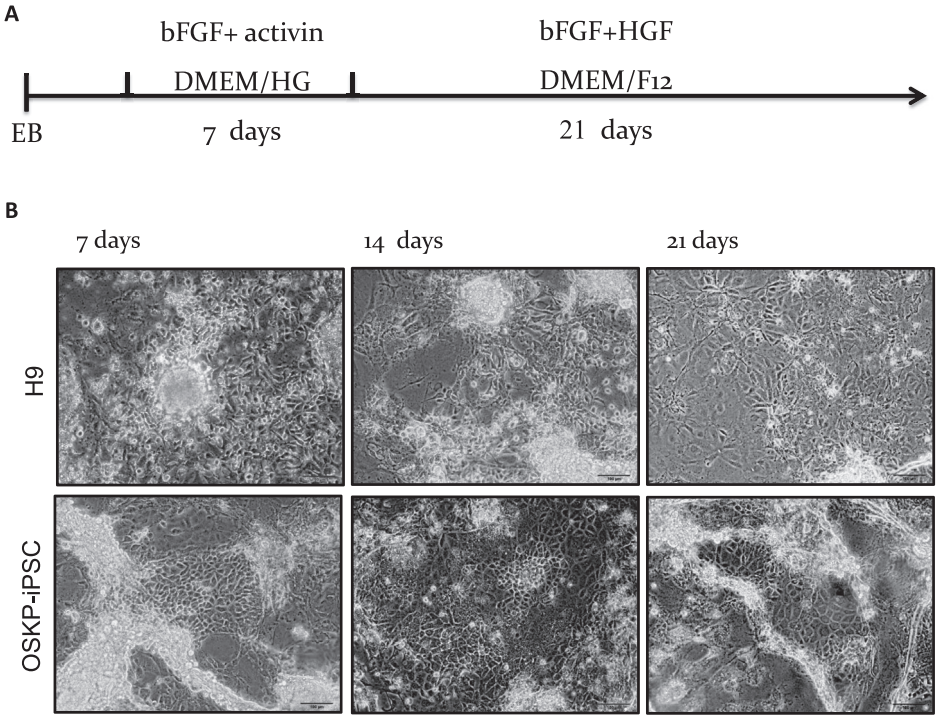

Fig. 2. **Differentiation of Oct4/Sox2/Klf4/Parp1-reprogrammed iPSCs into hepatocyte-like cells (OSKP-iPSC-Heps)** (A) Scheme illustrating the isolation of primary mouse tail skin fibroblasts and the generation of Oct4/Sox2/Klf4/Parp1-reprogrammed hepatocytes from 18-month-old aging mice. (B) Morphology changes of hESC H9 and OSKP-iPSC-Heps during the differentiation course at day 7, 14 and 21.

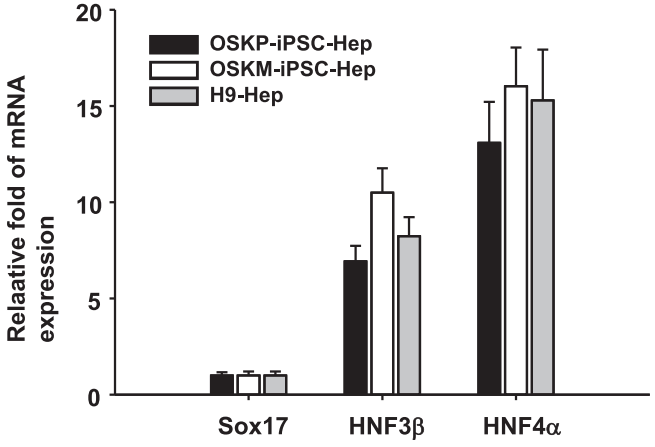

Fig. 3. **Conventional hepatic induction cocktail induces rapid maturation of OSKP-iPSC-Heps, OSKM-iPSC-Heps and H9-Heps.** Relative mRNA levels of Sox17, HNF3b, and HNF4a in OSKP-iPSC-Heps, OSKM-iPSC-Heps and human ESC H9-Heps at day 7 post-differentiation by using qRT-PCR.

population at day 21 (Fig. 5). Taken together, these findings demonstrate that the Parp1 could functionally replace c-Myc and support sufficient reprogramming with OSK. Furthermore, the substitution of c-Myc with Parp1 of OSKP-iPSC did not reduce the hepatic differentiation and functional maturity.

4. Discussion

Tumorigenesis is one of the major biosafety concerns in the application of stem cell therapy for treating human diseases. A recent study tried to test the feasibility of transplanting retinal pigment epithelial (RPE) cells differentiated from iPSCs into a patient with neovascular age-related macular degeneration. One year after transplantation, there was no evidence of lasting adverse effects in the patient, who received a transplant of a sheet of RPE cells derived from iPSC.[23,24] However, the biosafety of iPSC generation and improvement of iPSC quality are still the major issues for translational application. Forced expression of reprogramming factors can cause somatic cells to be reprogrammed into induced pluripotent stem cells (iPSCs), which possess potential for self-renewal and multi-lineage differentiation[8–11] and have been regarded as alternative sources for restorative cell therapy. In a report by Lapasset et al., it was demonstrated that modification of induction protocol Oct4/Sox2/Klf4/c-Myc by adding transcription factors Nanog and Lin28 could reprogram centenarian somatic cells into iPSCs.[12] c-Myc, a proto-oncogene that may cause genomic instability, has been linked to the risk of tumorigenesis, which may hinder the clinical applications of conventionally derived iPSCs.[13] Parp1 catalyzes PARylation and is a key effector involved in DNA repair, replication, transcription and genomic methylation.[16,17,25] In addition, Parp1 has been shown to regulate telomere length[26] and modulate telomerase activity by altering PARylation of telomerase reverse transcriptase (TERT).[27] Recently, it has been reported that Parp1 contributes to early-stage epigenetic modification during somatic cell reprogramming[20] and can promote reprogramming and maintain pluripotency.[21] In our study, we have shown that a four-factor cocktail (Oct4/Sox2/

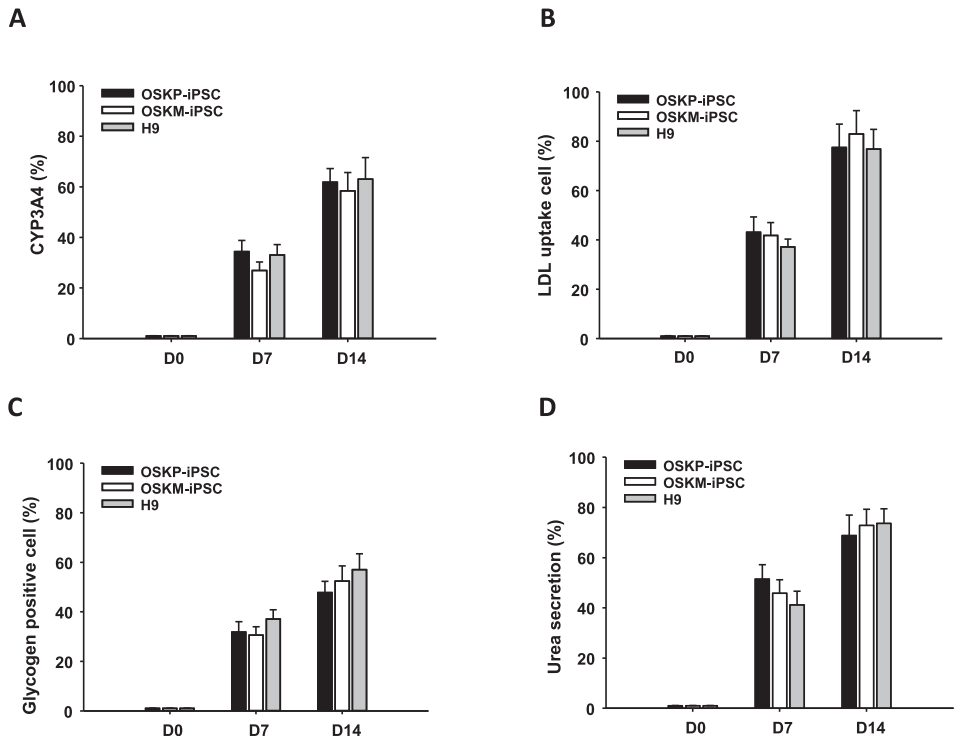

Fig. 4. **Comparison of hepatocyte-specific proteins and mature hepatocyte function between OSKP-iPSC-Heps, OSKM-iPSC-Heps and H9-Heps at various day post-differentiation.** Comparison of mature hepatocyte functions including CYP3A4, LDL uptake, glycogen synthesis and urea secretion between OSKP-iPSC, OSKM-iPSC and H9 cells at day 0, day 7 and day 14 post-differentiation.

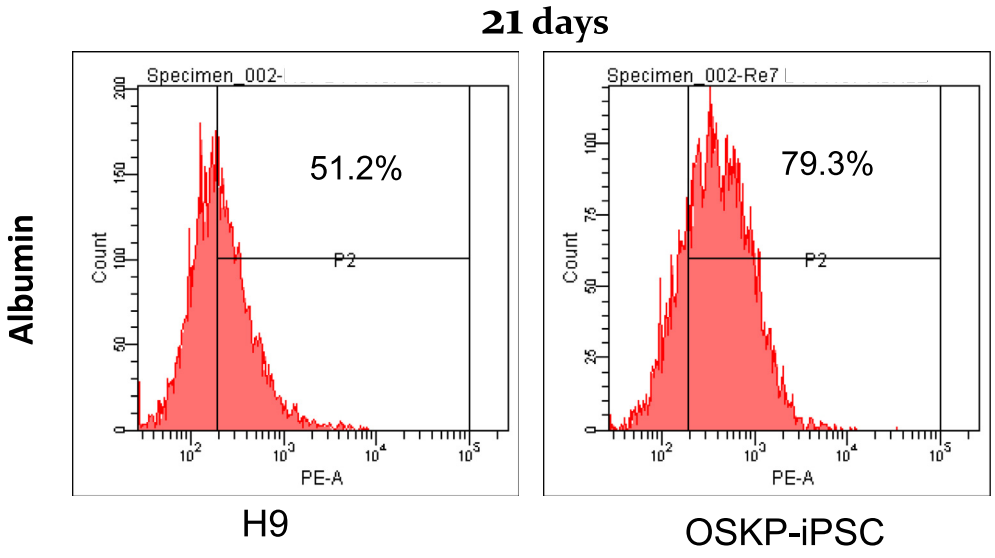

Fig. 5. **Comparison albumin expression in OSKP-iPSCs and ESC H9 cells.** The albumin expression of OSKP-iPSC-Heps and H9-Heps measured by the flow cytometry at day 21 post-differentiation.

Klf4/Parp1; OSKP) could successfully reprogram fibroblast into iPSCs in the absence of exogenous c-Myc gene. The resultant OSKP-iPSCs could undergo hepatic-specific differentiation and generate OSKP-iPSC-Heps, which exhibited better hepatoprotective capacity.

Importantly, our findings indicated that Oct4/Sox2/Klf4/Parp1(OSKP)-induced reprogramming can generate iPSCs that formed smaller teratomas than the conventional Oct4/Sox2/Klf4/c-Myc-induced iPSCs. After hepatic-specific differentiation, OSKP-iPSC-Heps expressed a prominent antioxidant defensive activity to significantly scavenge the accumulation of oxidative substances. Consistently with the previous reports,[28–31] we showed that OSKP-iPSC-Heps appeared to be more resistant to the oxidant insults (data not shown). In addition, comparing with other type of iPSC-derived hepatocytes, OSKP-iPSC-Heps exhibited the high

qualities of liver characteristic and functions, including high capability of anti-oxidative stress, anti-pro-inflammatory cytokines and anti-fibrogenic changes. With the consideration of biosafety in application, the use of iPSC-derived cells in therapeutics is now facing additional challenges, associated with reprogramming protocols involving proto-oncogenes.

Therefore, in the future, more data related to the patient response to implanted iPSC-differentiated hepatocyte will be required to validate the future clinical utility of patient-specific iPSC-based therapy in different liver disease cohorts.

Acknowledgments

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